A study of normal and abnormal forms of prion protein in the peripheral blood and tissues of patients with variant Creutzfeldt-Jakob disease

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Declaration

I declare that this thesis has been composed entirely by myself and that the work presented herein is my own, except where otherwise stated.

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Abstract

A recent report of a case of variant Creutzfeldt-Jakob disease (vCJD) possibly transmitted by blood transfusion implies infectivity in the blood of vCJD patients. There is therefore an increasing need for screening tests able to identify infected individuals during the incubation phase of disease to ensure the safety of transfused blood products and reduce the incidence of iatrogenic disease transmission.

Firstly this thesis assesses the potential of the detection of PrP^{c} as a surrogate marker for CJD by an analysis of blood from vCJD patients, sCJD patients, non-CJD neurological controls and healthy adults, PrP^{c} was measured by DELFIA and cell-associated PrP was measured by flow cytometry. There are differences in free and cell-associated PrP found in blood of CJD patients and control groups, some of which may be useful as surrogate markers of disease. In addition the development of a DELFIA based test designed for the detection of the disease-associated PrP^{Sc} in human peripheral blood is the other main focus of research studies detailed within this thesis. The use of atomic dialectric resonance spectroscopy analysis techniques to explore potential differences in frequency and atomic resonance which may allow identification of characteristics distinct to vCJD peripheral blood samples have also been assessed.

Abbreviations

Ab	Antibody
ADR	Atomic Dielectric Resonance
BC	Buffy Coat
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
C	Celsius
CB	Coating Buffer
CDI	Conformation-Dependent Immunoassay
CJD	Creutzfeldt-Jakob disease
CNS	Central Nervous System
CPM	Counts Per Minute (Eu^{3+})
CSF	Cerebrospinal fluid
CWD	Chronic Wasting Disease
DELFIA	Dissociation Enhanced Lanthanide fluoroimmunoassay
DH_2O	Distilled water
EDRF	Erythroid differentiation-related factor
EEG	Electroencephalogram
FFI	Fatal Familial Insomnia
FTIR	Fourier Transformed Infrared
GdnHCL	Guanidine Hydrochloride
GdnThio	Guanidine Thiocyanate
GFAP	Glial fibrillary acidic protein
GPI	Glycosylphosphatidylinositol
GSS	Gerstmann-Straussler-Scheinker Disease
IP	Intracranial
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
MeOH	Methanol

MAb	Monoclonal antibody
µMol/L	Micromoles per Litre
NaCL	Sodium Chloride
NaOH	Sodium hydroxide
NaPTA	Sodium Phosphotungstic Acid
nMol	Nanomolar
NOG	n-octyl β-D-glucanopyranoside
O/N	Over Night
PAGE	Poly-Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PK	Proteinase K
PMSF	Phenylmethanesulfonyl fluoride
PPP	Platelet Poor Plasma
PrP	All reference to the protein
PrP^c	Cellular Prion protein
PrPres	PK resistant prion protein
PrP^{Sc}	Scrapie Prion protein
PT	Platelet
PVDF	Polyvinylidene Fluoride
RBC	Red blood Cells
RCF	Relative Centrifugal Force
RNA	Ribonucleic Acid
RPM	Revolutions per minute
RT	Room Temperature
SBO	Specified Bovine Offal
sCJD	Sporadic Creutzfeldt-Jakob disease
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline + 0.1% Tween 20
TME	Transmissible mink encephalopathy
TSE	Transmissible Spongiform Encephalopathy
vCJD	Variant Creutzfeldt-Jakob disease

V	Volts
WB	Whole Blood
W/V	Weight to volume

Chapter 1: Introduction

1.1 TSEs: Overview

The transmissible spongiform encephalopathies (TSEs) are a family of transmissible neurodegenerative diseases including CJD and Kuru in humans, scrapie in sheep and goats, chronic wasting disease (CWD) in deer, and bovine spongiform encephalopathy (BSE) in cattle. They are characterised clinically by dementia, ataxia, and myoclonus, and histopathologically by astrogliosis, dendritic spongiosis, and neuronal loss [1].

A key event in the pathogenesis of TSE diseases is the post-translational conformational conversion of cellular prion protein from its soluble protease sensitive isoform (PrP^{c}) to the pathological protease resistant scrapie isoform (PrP^{Sc}). Frequently neuroinvasion and accumulation of PrP^{Sc} into the central nervous system is responsible for, and is a feature of the pathology of the condition. Consequently PrP^{Sc} is used as a surrogate marker for and is thought to be synonymous with infectivity [2-4]. TSEs are characterised by long incubation periods that vary from months in experimental animal models to many years in certain forms of human CJD.

1.2 Prion Protein and the Prion theory

In humans the normal cellular isoform of prion protein PrP^{c} is a 35-kDa glycosylphosphatidylinositol (GPI)-anchored membrane glycoprotein expressed predominantly on neurones but also on many other cell types and tissues including

all blood cells with the exception of eosinophils [5-8]. PrP^c can also be detected in a soluble form in blood and plasma, known to be released from platelets and vascular endothelial cells, with the largest cellular component expressed on mononuclear cells and platelets [9,10]. Its physiological function however is not clearly understood. PrP^c is known to interact with glial fibrillary acidic protein (GFAP), and immunoprecipitation and cell culture studies have demonstrated interactions with calnexin, calreticulin, laminin, heat shock proteins, and heparin sulphates. PrP^c may have roles in synaptic function, copper transport, superoxide dismutase activity, signalling, and cell survival and differentiation.

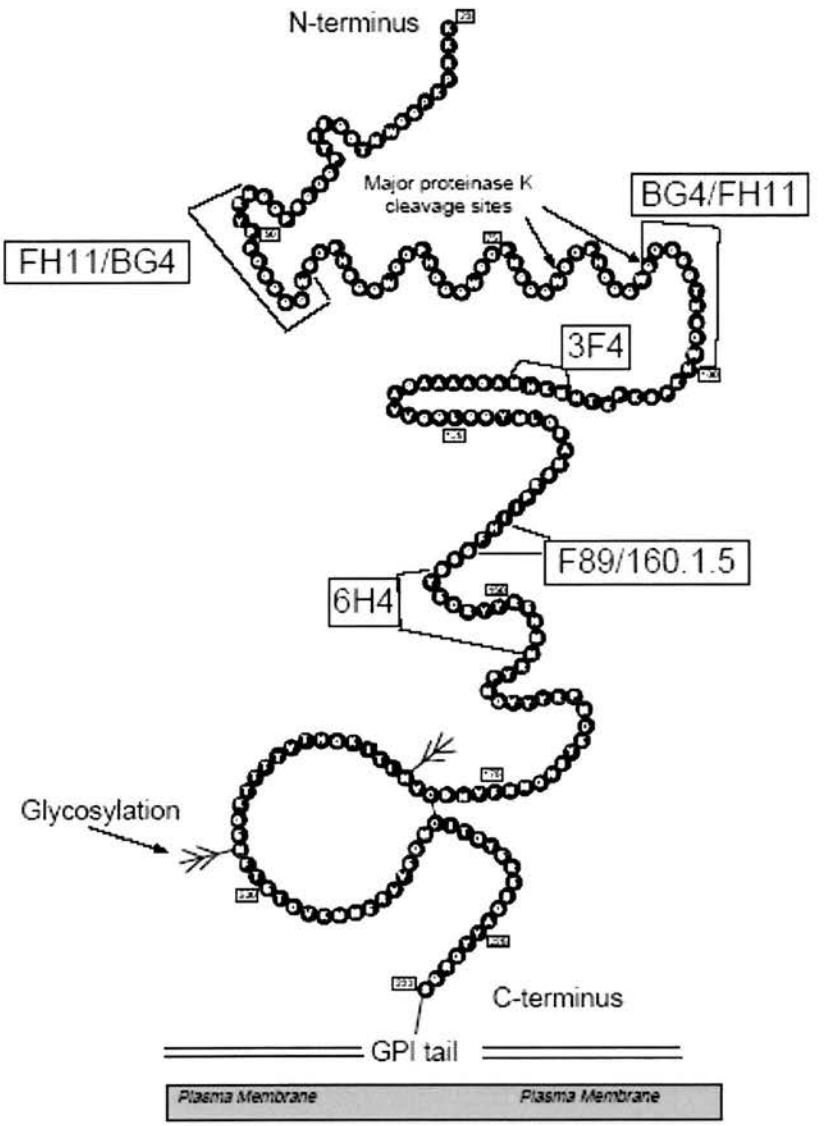
The protein has two sites of asparagine linked glycosylation at residues 181 and 197, and a disulphide bridge between cysteine residues at 178 and 213 [11]. NMR studies of recombinant human PrP found that there is a C-terminal core region (124-231) folded into three α-helices. The disulphide bond bridges cysteine residues on the second and third helices. There are also two anti-parallel beta sheet strands. The N-terminus (23-124) is relatively unstructured and is highly flexible [12], (Figures 1, 2). The disease associated prion protein PrP^{Sc} was found to be insoluble in non-ionic and non-denaturing anionic detergents, had a propensity to form aggregates, and was partially resistant to proteolysis with proteinase K (PK) [2]. The protease resistant core denoted PrP27-30 assembles into amyloid polymers [13]. No chemical modification was found to be associated with the formation of PrP^{Sc} from PrP^c [14], suggesting that conformational differences may be responsible for different properties of the two forms of PrP. Analysis of both PrP^c and PrP^{Sc} by fourier-transform infrared spectroscopy and circular dichroism measurements illustrate differences in

conformation. PrP^{c} was found to have 42% α-helix and very little if any β-sheet (3%), whereas PrP^{Sc} was found to have high levels of β-sheet (43%) and less (21%) α-helix, these differences are illustrated in Figure 3. It would appear therefore that the conversion of one isoform to another is based upon conformational changes which appear to be fundamental to prion propagation [15].

Figure 1

Adapted from [12], 3D structure of human prion protein, the helices are shown in red, the β strands in cyan, unstructured c terminus in yellow, and the flexible tail in yellow dots.

Figure 2

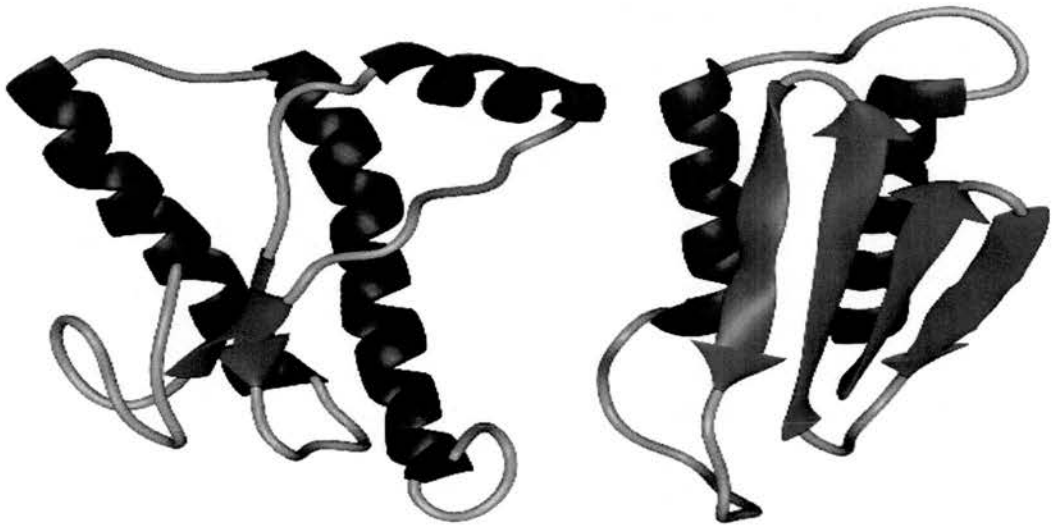


Human Prion Protein from [16]. The amino acid sequence is shown with single letter code for the mature protein after removal of the N-terminus signal peptide and the C-terminus signal sequence. Main proteinase K cleavage sites are shown with arrows. The single disulphide bond is shown between C178 and C213. The two potential glycosylation sites at N181 and N197 are illustrated with branched arrows. GPI indicates the glycosyl phosphatidylinositol anchorage to the cell membrane attached via S230. The epitopes recognized by some widely available monoclonal antibodies are identified. FH11 and BG4 Institute for Animal Health Compton Laboratories; 3F4 ± Senetek Inc: 6H4 Prionics; F89 as MAI-750 Affinity Bioreagents Inc.

The exact mechanism of conversion remains controversial. The protein only hypothesis states that PrP^{Sc} is propagated by the conversion of PrP^{c} molecules to an altered conformation; two models have been proposed. Firstly PrP^{Sc} may act as a template upon which PrP^{c} is refolded into a nascent PrP^{Sc} molecules through a process facilitated by another protein [3] often referred to as protein X [17].

The template conversion theory is supported by a large amount of empirical evidence which include experiments where hyper and drowsy hamster transmissible mink encephalopathy strains were able to convert PrP^{c} into distinct sets of protease resistant PrP^{Sc}-like products in a cell-free system [18]. This suggests that three-dimensional structure could be the molecular basis for disease strains. These findings were supported by transmission studies using brain extracts from FFI and familial CJD (E200K) and sCJD to transgenic mice expressing a chimeric human-mouse PrP gene. Characteristic deglycosylated protease resistant fragments of 19-Kda for FFI and 21-Kda for familial and sCJD brain material were faithfully reproduced when mice brain extracts were analysed 200 days post inoculation [19]. Additional transmission studies investigating incubation periods and neuropathology found that a scrapie strain when transmitted to Syrian hamsters had distinct properties from the same strain passaged through transgenic mice with chimeric Syrian hamster / mouse prion protein genes suggesting that strain diversity enciphered in conformation seems to be restricted by PrP^{c} substrate, while supporting the propagation of PrP^{Sc} by template interaction [20]. Conformational analysis of scrapie strains by equilibrium unfolding found different strains have unique conformations which exhibit different susceptibilities to proteases [21].

Figure 3



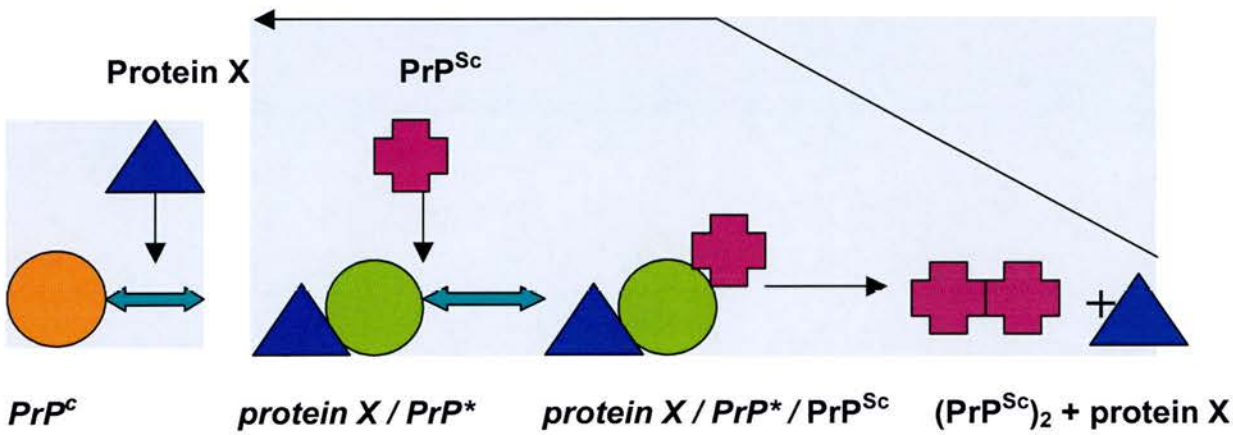
PrP^{c} PrP^{Sc}

Diagram illustrating conformational differences between PrP^{c} and PrP^{Sc}

Figure 4

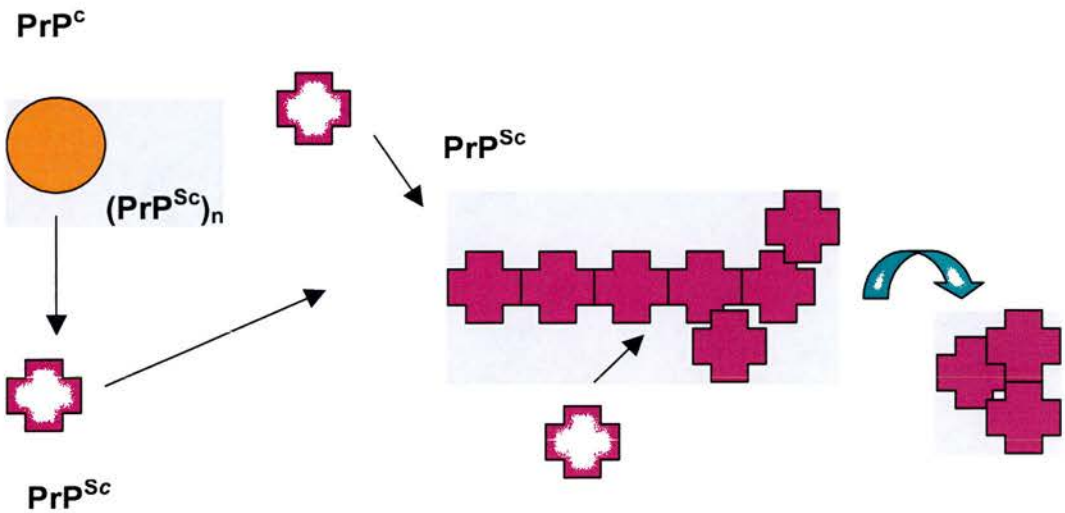
a) Template conversion theory

PrP^{c} binds to an unknown chaperone which initiates conformational change in PrP^{c}. PrP^{Sc} then binds to this conformationally altered PrP. The incoming PrP^{Sc} acts as a template refolding PrP* to PrP^{Sc} which results in the formation of a dimer of PrP^{Sc}, protein X is released and is available once again to reinitiate conversion events.*



b) Nucleation theory

Both PrP^{c} and PrP^{Sc} exist in equilibrium, monomeric PrP^{Sc} nucleates into aggregates. PrP^{Sc} molecules may dissociate from the aggregate to initiate seeding in other areas



The second model states that the conversion process may be facilitated by nucleation of unfolded PrP^{c} where the hydrophobic 96-112 amino-acid sequence is exposed, see Figure 4. This region was found to be exposed in PrP^{c} but hidden in PrP^{Sc}, and has been found by peptide deletion and antibody mapping to be essential to conformational change associated with the formation of PrP^{Sc} [22-24]. Peptide aggregation experiments suggest that this sequence may initiate the formation of stable aggregates from monomeric PrP^{Sc} molecules via a nucleation-dependent self-assembly mechanism [25]. It is also thought that parts of the aggregates may break from the larger assembly and may be capable of initiating PrP^{Sc} seeding in distinct areas.

What is certain is that PrP^{Sc} facilitates the conversion of normal PrP^{c} and there is compelling evidence provided by in vitro conversion experiments that describe the amplification of PrPres. An excess of PrP^{c} is utilised as a substrate for conversion, and is converted to PK resistant forms by minute quantities of PrP^{Sc} [26,27]. Aggregates formed when PrP^{c} is incubated with PrP^{Sc} are disaggregated by sonication thereby producing smaller aggregates and amplifying sites available to facilitate additional conversion events. Recent evidence has indicated that host encoded RNA (>300 nucleotides) cofactors are required for the amplification of PrPres in vitro since the use of RNase prevented the production of PrP^{Sc} dose dependently [28]. PrPres was amplified six fold following incubation of uninfected hamster brain homogenate with infected hamster brain homogenate. When homogenates were treated with RNase to deplete all single stranded RNA no amplification occurred; there was 24-fold amplification with the addition of RNA.

1.3 The Viral theories

Although PrP^{Sc} is thought to be the infective agent, and infectivity has been found to co-purify with PrP^{Sc} preparations, not all PrP^{Sc} is associated with infectivity [29]. A recently described murine model (101L) of TSE disease with leucine mutation at amino acid 101 exhibits no detectable PrP^{Sc} but brain material remains infectious to homozygous 101L mice with extremely short incubations periods [30]. Similar results have been obtained in the transmission of BSE to mice [31].

The inability to generate infectivity in protease resistant PrP^{Sc} converted in-vitro from PrP^{c} [32] and the fact that classical amyloid diseases, which share protein misfolding as a feature of pathogenesis, are not easily experimentally transmissible make many sceptical of the 'protein only' explanation for TSE infectivity.

The exact nature and structure of the infective agent is far from clear and critics of the prion theory doubt whether structure and conformation alterations are sufficient to encode the complex biological and biochemical properties of different TSE strains. PrP^{Sc} may propagate through interaction with an as yet unidentified additional infectious agent. The virino theory supports the existence of a host independent replicable information molecule which is associated with host prion protein and encodes TSE strain characteristics [4,33-35]. The virus hypothesis suggests that the infectious agent and genetic information is carried by a conventional virus [36]. In a recent publication [37] an anti-DNA antibody 'OCD4' and a single stranded DNA binding protein 'g5p' are described which bind to PrP^{Sc} and disease associated protein but not PrP^{c}. Since the antibodies are raised with a nucleic acid immunogen,

and 'g5p' is a DNA binding protein, their interaction with PrP^{Sc} would imply that the disease-associated protein is associated with DNA or a DNA associated molecule. This has important implications in understanding the associations and interactions of PrP^{Sc} and may provide the evidence that strain characteristics are in-fact encoded by a nucleic acid as implied in the virino hypothesis. It will be interesting to discover whether the protein-nucleic acid species retain infectivity following immunocapture and isolation from infected brain tissues with 'OCD4' and 'g5p', whether these are specific interactions between antibody and a nucleic acid component of a prion protein complex, and whether the anti-DNA antibody recognises PrP^{Sc}.

1.4 Species barrier

Experiments in animals illustrate that a species barrier reduces the ease of TSE transmission between species. This causes an increase in the length of the incubation period prior to the appearance of clinical symptoms, and causes a reduction in the number of inoculated animals that succumb to clinical infection. The species barrier is affected by polymorphisms in the sequence of host PrP which may not be compatible with the PrP sequence of the donor, prion strain and the route and dose of infective agent. What is interesting is that on secondary passage through the same species there is a reduction in the barrier to transmission, a reduction in incubation period and strain characteristics become more consistent. Transgenic mouse models have been developed where mice express human or bovine PrP with the host mouse PrP or in isolation. Since these models express PrP^{c} that is compatible with the inoculated agent this removes the species barrier causing a reduction in incubation periods and allows strain typing of human TSEs in mouse models. These models

suggest that PrP^c is the major determinant in species barriers. Transgenic mice such as these which express human PrP^c have been used for strain typing of BSE and vCJD. Similar strain typing support the view that vCJD and BSE are caused by exposure to the same agent strain [38,39]. PrP^c is not the only determinant of the species barrier since transgenic mice expressing human PrP do not exhibit a barrier to infection with sCJD, however a barrier is apparent in transmission of vCJD [40] despite the fact that donor and recipient PrP have the same codon 129 genotype and identical primary structure.

1.5 Animal and experimental TSEs

1.5.1 Natural and experimental scrapie in sheep

Scrapie was first described in 1755 in the British parliament as a fatal disease affecting sheep. In 1936 transmissibility was demonstrated by the successful transmission of scrapie to two healthy sheep by intraocular inoculation with a 1-2 year incubation period [41]. Scrapie is endemic in all countries that farm sheep, with the exception of New Zealand and Australia. Clinical signs include odd behavioural patterns: animals lie down and have abnormal gait, bite and rub themselves against objects causing pruritis. The infection in the latter stages affects the animals' ability to feed and sheep become emaciated and die.

Scrapie pathology is characterised by vacuolation of the grey matter neurophil; vacuolar lesions in the neocortex; neuronal degradation; astrocytosis; amyloidosis and the deposition of PrP^{Sc}.

The exact mode of transmission of natural scrapie between sheep and flocks is unknown. The oral route of transmission is suspected to be the most likely route. The susceptibility and development of clinical scrapie in sheep is strongly associated with certain genetic types, initially scrapie was thought to be a genetic disease, it is clear however that exposure to the infectious agent is essential for the development of scrapie [42] and that the PrP gene sequence has significant effects upon disease susceptibility. This is primarily controlled by amino-acid sequence at codons 136,154, and 171. Since sheep carry two chromosomes with the PrP gene allele, there are many possible different combinations, despite this only 5 alleles are commonly found in sheep: VRQ, ARQ, ARR, AHQ, ARH of these the VRQ/VRQ genotype is at most risk of developing scrapie, and ARR/ARR the most resistant

genotype. Certain genotypes are restricted to certain sheep breeds, however in light of the strong associations of particular genotypes with disease susceptibility the UK national scrapie plan aims to breed sheep to ensure a higher frequency of sheep with the less susceptible genotype to reduce the risk of infection.

1.5.2 Bovine spongiform encephalopathy (BSE)

BSE was first recognised in Britain in November 1986 and led to an epidemic which grew considerably until the early 1990s. The disease affects animals 4-5 years old, clinical disease lasts several weeks and is fatal. Clinical effects include abnormalities in posture and gait, with behavioural abnormalities.

BSE was considered an extended common source epidemic and there was no evidence of between cattle transmission. All cases of BSE were found in herds which used a commercially produced compound feed which contained meat and bonemeal. Material from scrapie infected sheep is the most likely culprit for the infective agent [43]. The epidemic was sustained by the presence of BSE infected cattle material being recycled in feed and cattle becoming infected by eating the infected remains of other cattle. The risk of exposure was thirty times greater for calves than adult cows [43]. The sudden emergence of BSE is thought to be caused by a change in the rendering processes in the late 1970s early 1980s which led to an increase in the levels of the infective agent during the processing of animal by-products into meat and bonemeal as a result of using lower temperatures and solvents, thereby allowing the agent to infect cattle [44].

The exact origin of the infective material is unknown; one theory is that the agent arises from the presence of scrapie infected sheep material in feed. The neuropathological characteristics of BSE are very similar to scrapie [43], the incidence of scrapie increased significantly in the 1980s and therefore suggests that its presence in feed may have risen accordingly. Britain had the highest ratio of sheep to cattle during this time, so although other countries were employing similar rendering processes the level of sheep material in the composite feed was highest in Britain. Alternative explanations include a sporadic event much like sCJD which occurred by spontaneous mutation in a cow causing BSE in other cattle when the carcass was processed and included in feed, however this would not explain why the emergence of BSE was restricted to Britain and why there were no cases prior to 1985. The BSE enquiry favoured the spontaneous mutation hypothesis, and thought this to be a more likely cause than that of scrapie infected feed. The Horn enquiry did not rule out scrapie as the culprit, it established that during the 1970s Britain began to introduce meat and bonemeal into calf feed from 2 weeks old and given that they are more susceptible to infection from contaminated feed this may have been key to the BSE epidemic [45]. In Australia there was a similar change to feed meat and bonemeal to calves however BSE did not arise in Australia perhaps because the country is scrapie free. BSE however is biologically and molecularly distinct from all scrapie strains, which would appear to disprove the scrapie link [46].

In an attempt to control the BSE outbreak a ban in 1988 was enforced to prevent cows eating cow derived feeds. This feed ban had a dramatic effect upon the number of BSE cases which by 1992 were falling by a rate of 40% per year. The delayed

effect of the ban was consistent with the long incubation period for BSE, and confirmed that contaminated feed had indeed been responsible for disease transmission. Unfortunately BSE cases continued to occur after the ban, possibly a result of cross contamination of feed intended for pigs that still contained cattle derived meat and bonemeal [47]. Additional preventative measures banning the presence of any mammalian protein in animal feed were introduced in the UK in 1996. This was largely a response to the identification of vCJD in the same year. These measures were introduced in the EU in January 2001. To date 69 cases of BSE in animals born after the ban of 1996 have been detected by passive or active surveillance [48].

Since BSE has emerged several policies have been introduced in order to ensure the safe consumption of British beef. In 1989 a ban on specified bovine offal (SBO) was introduced to prevent tissues from entering the human food chain. Infectivity studies of scrapie in sheep had identified the presence of the infective agent in the brain, spinal cord, tonsil, thymus, spleen and intestines [49]. This was followed by a policy that no British cattle over the age of 30 months should be consumed, as advised by the Spongiform Encephalopathy Advisory Committee in May 1996. This policy is now under review considering the vast reduction of BSE in the UK. BSE has occurred outside the UK due to the export of cattle derived products and meat and bone meal contaminated cattle feed. Table 1 illustrates the numbers of confirmed BSE cases worldwide. A recent case of BSE has occurred in a dairy cow in Washington State USA. This animal was subsequently confirmed to have been imported from a Canadian herd in Alberta Canada, in 2003 Canada confirmed its

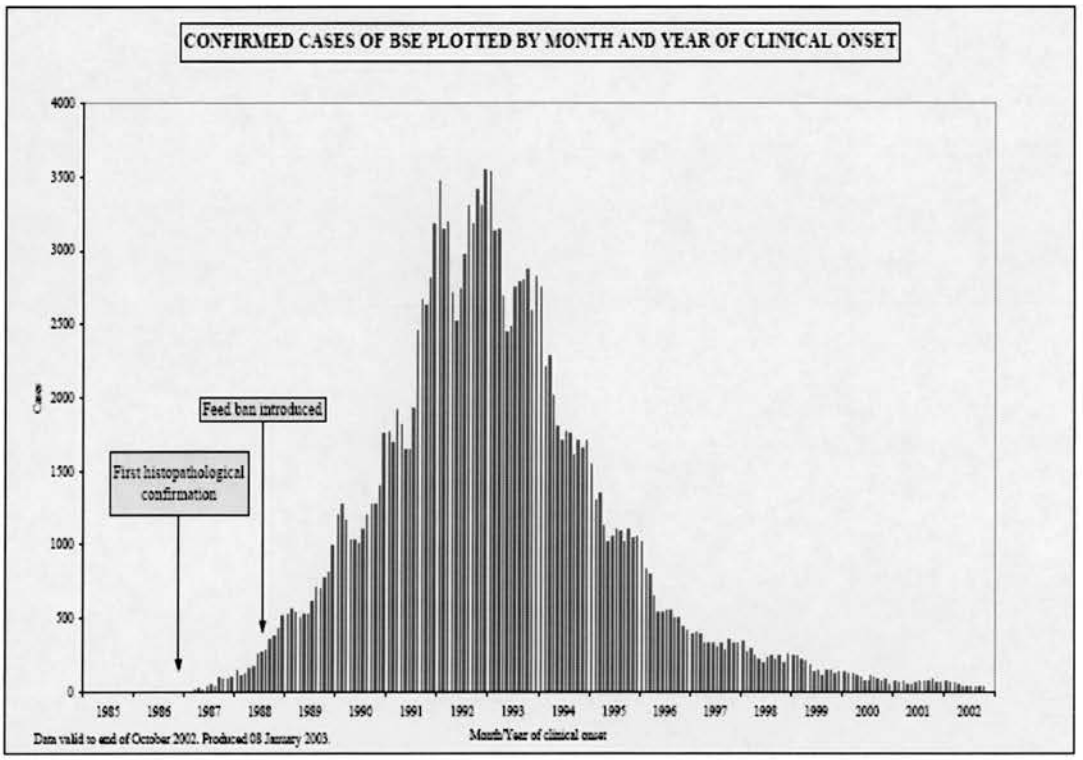
first case of BSE in a cow from Alberta, it is unknown whether the two cases are connected.

Table 1

BSE in European Union Countries 2004				
	2004	2003		Total since 1987
Austria	0	0		1
Belgium	11	15		129
Czech Republic**	7	4		17
Cyprus**	0	0		0
Denmark	1	2		14
Estonia**	-	-		-
Finland	0	0		1
France	54	137		951
Germany	65	54		369
Greece	0	0		1
Hungary**	-	-		-
Ireland	126	182		1488
Italy	7	31		126
Latvia**	0	0		0
Lithuania**	0	0		0
Luxembourg	0	0		2
Malta**	0	0		0
Netherlands	6	19		77
Poland**	11	5		22
Portugal	92	133		954
Slovak Republic**	7	2		19
Slovenia**	2	1		5
Spain	137	167		538
Sweden	0	0		0
UK (GB & Northern Ireland)*	398	611		182807

Figures as at 31 December 2004.
* UK figures include Great Britain figures for passive and active surveillance, and a Northern Ireland total of 34.
** Countries that joined the EU on 1 May 2004.
Sources: for UK: Defra, DARD and Braakman.
Data compiled by Food Standards Agency (http://www.food.gov.uk/).

Figure 5



Confirmed cases of BSE in the UK. Sourced from (http://www.defra.gov.uk/)

1.5.3 Chronic wasting disease (CWD)

CWD was first described in 1980 as a spontaneously occurring form of spongiform encephalopathy affecting mule and black tailed deer in captive populations in Colorado and Wyoming [50]. The disease was subsequently found to affect both captive and free ranging deer populations and rocky mountain elk. CWD causes widespread spongiform change in the grey matter, neuronal loss, astrocytic hypertrophy and hyperplasia and the deposition of amyloid plaques [51]. PrP^{Sc} is detectable in the brain and lymphoreticular tissues of infected animals [52]. Clinically the disease is characterised by behavioural problems, chronic weight loss, and death from two weeks to eight months after infection [50]. The disease is infectious and

contagious, however the specific mode of transmission remains unidentified. Contaminated foodstuffs are not responsible, lateral transmission is important to the spread of disease. The disease may be transmitted via the faeces or saliva, the infectious agent has been detected in lymphoid tissues of the alimentary tract, and contaminated pasture has been responsible for further outbreaks.

The disease origin is unknown, it is possible that CWD may be an adapted scrapie strain, although strain typing and biochemical analysis does not suggest scrapie PrP^{Sc} is related to the form found in CWD [53]. Other explanations to explain the emergence of CWD are that it may have been a spontaneous sporadic incident, or a genetic form arising in deer and subsequently transmitted amongst the deer population and to other species.

1.5.4 Transmissible mink encephalopathy (TME)

TME was first recognised in the 1940s in Wisconsin and Minnesota USA. The disease has arisen in farmed mink in other countries including Russia, Canada, and Germany but disease outbreaks remain rare [54]. The cause of TME is uncertain, initially epidemiological evidence indicated that scrapie contaminated foodstuffs may be the source of outbreaks, however transmission experiments found that mink were only susceptible to scrapie by intracranial (IC) inoculation and not by ingestion [55]. Mink were found to be highly susceptible to transmission of disease by both the oral and IC route if the disease was firstly passaged through cattle [56]. TME has been well characterised by transmission to hamsters where it exhibits two different strains: hyper and drowsy which have different incubation periods and whose PrP^{Sc} has different biochemical properties [18]. It may be plausible that in order for the agent to

become infectious to mink it required prior propagation in distinct animal species. Clinically the disease is characterised by increased aggression, ataxia, hyperesthesia, and has an incubation period of 7-12 months [54]. Histopathology of the disease includes widespread vacolation of the neurophil, neuronal cell death and astrocytosis.

Because TME has arisen as a result of oral exposure to infected feed by either a scrapie or BSE type agent, it is unlikely to pose a future problem due to the exclusion of sheep and cattle from feedstuffs.

1.6 Human TSEs

In humans CJD can be divided into four groups: sporadic, familial, iatrogenic, and variant.

CJD may occur sporadically with an incidence of between 0.5-2 cases per million population a year typically affecting older people between the ages of 55 and 80. Cases of familial prion diseases are a result of autosomal dominant mutations in the prion gene *PRNP* open reading frame; they often clinically resemble sporadic forms of the disease.

CJD has been transmitted iatrogenically via the use of contaminated neurosurgical equipment, by dura mater, corneal transplants, and by contaminated cadaveric gonadotropic growth hormones[57].

The National CJD Surveillance Unit first identified new variant Creutzfeldt-Jakob disease (vCJD) in 1996 as a novel transmissible spongiform encephalopathy (TSE)[58]. vCJD is characterised by its specific neurological profile, young age at onset, unusual clinical course as compared with sCJD, the absence of mutations within the *PRNP* gene and no recognised risk factors for iatrogenic CJD. There is compelling evidence that vCJD results from the consumption of beef infected with BSE.

1.6.1 Sporadic CJD

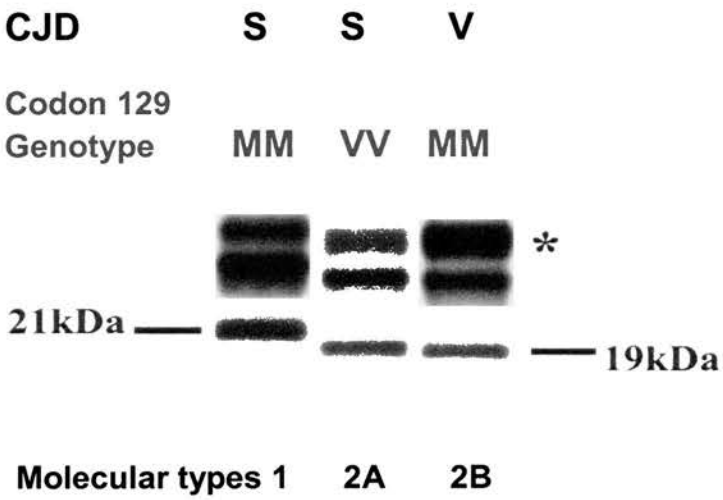
Sporadic CJD is a rapidly progressive multifocal dementia, which may feature monoclonus, cerebellar ataxia, pyramidal and extrapyramidal signs, and cortical blindness[59]. Sporadic CJD accounts for ~ 85% of all detected human prion disease [60,61], it has a uniform world-wide incidence of 1 case per million per year with an average age at onset of 60 years.

The cause of sporadic CJD is unknown but it is thought that it may arise from spontaneous PrP^{Sc} production, somatic mutation, post-translational modification of *PRNP*, or unidentified prion exposure [59]. Neuropathologically the condition can cause spongiform change, neuronal loss, and astrocytosis in the cerebral cortex, occipital lobe, thalamus and cerebellar cortex. PrP^{Sc} deposition is usually detected in areas of spongiform change. A polymorphism at codon 129 of prion protein gene which encodes either methionine or valine amino-acid residues exerts an influence upon the susceptibility of Caucasians to sporadic CJD. Heterozygotes are significantly protected against developing sporadic CJD[62-64]. A great deal of clinical heterogeneity can be observed in sporadic CJD, and although the causes are not fully

understood it is likely that a combination of codon 129 genotype and prion strain contribute towards this.

Strain typing studies using transgenic mice expressing human PrP support the suggestion that strain characteristics are encoded in the conformation and glycosylation patterns of PrP^{Sc}, which are maintained on passage[40]. Analysis by western blot of infected mouse brain tissue treated with PK allows distinction between strains based on the pattern of fragment migration due to different N-terminal cleavage sites, and also glycosylation ratios of diglycosylated, monoglycosylated and unglycosylated PrP banding patterns. These differences are due to differences in glycosylated PrP species. Such strain typing has allowed the identification of PrP isotypes which have different phenotypes of sporadic CJD[40,65-68] and are associated with specific clinical and pathological criteria. Parchi [65] identified two molecular types of PrP^{Sc} in sporadic CJD. A type 1 western blot pattern was found to associate with methionine homozygosity at codon 129 and with typical sporadic CJD disease phenotype. Type 2A blotting pattern was associated with all genotypes at codon 129 and with atypical rarer clinical variants of sporadic CJD[65,68]. These types along with type 2B, which shares the same fragmentation as type 2A but has a different glycoform ratio due to its heavy diglycosylation (vCJD), are used to characterise acquired forms of CJD including vCJD (see Figure 6).

Figure 6



*Western blot analysis of 2 sCJD types and vCJD (Adapted and reproduced with permission from Dr Mark Head). The blot illustrates the banding patterns of diglycosylated (top band), monoglycosylated (middle band), and unglycosylated (bottom band) prion protein after treatment with PK which are associated with sporadic and variant forms of CJD. Type 1 and 2A patterns are associated with sCJD of different genotypes at codon 129 and there are differences in the migration of the unglycosylated band. The monoglycosylated band is the most prominent in type 1 sCJD. vCJD * binding pattern is similar to type 2 sCJD but the diglycosylated band is much more prominent.*

An alternative nomenclature for the classification of both sporadic and acquired forms of CJD has been proposed where there are 6 types used to categorise both sporadic and acquired CJD in humans [59]. In this classification CJD types share the same association with codon 129 genotype as those proposed by Parchi et al [40] but Hill et al [59] claim to have identified additional types for iatrogenic and sCJD based upon differences in molecular mass and fragment sizes when samples are analysed by western blotting techniques. Subsequent to these findings is a report that a significant heterogeneity in pH among CJD brain homogenates exists which in turn influences the size of the PrP^{Sc} core generated by protease digestion. By demonstrating that the heterogeneity of human PrP^{Sc} type 1 within specific groups (MM or MV subjects) strictly depends on pH variations among CJD brain homogenates, this study provides a reasonable technical explanation for the PrP^{Sc} molecular heterogeneity [69].

1.6.2 Iatrogenic CJD

The first case of iatrogenic CJD was reported in 1974 in the recipient of a corneal graft from a donor who had died of unknown CJD [70]. The major causes of iatrogenic CJD include contaminated human cadaveric human growth hormone, and dura mater grafts which were first recognised as risks in the 1980s [71,72]. Data included in a recent review of iatrogenic CJD examined 267 cases in total of which 139 were infected through human cadaveric growth hormone, 114 by dura mater, of the remaining cases <5 in each group were due to transmission by corneal transplant, contaminated EEG or neurosurgical equipment [73], or through contaminated gonadotropin [57]. The majority of cases of iatrogenic CJD caused by contaminated

growth hormone (>50%) have occurred in France, similarly >50% of the cases of CJD transmission due to dura mater graft have occurred in Japan.

Iatrogenic CJD due to contaminated human growth hormone has been best characterised by studies in France [74]. Pooled human growth hormone infected by at least one cadaver with preclinical CJD caused the majority of these cases. Of 1700 patients receiving human growth hormone 74 developed CJD (4.4%), the mean incubation period was 10 years, the long duration most likely due to the low levels of infectious dose and route of administration.

The codon 129 M/V polymorphism was found to affect incubation period in certain forms of iatrogenic CJD, 80% of iatrogenic CJD cases were homozygous, Methionine homozygotes were found to be the most susceptible group in dura mater cases. In cases of growth hormone transmission a notable 32% were valine homozygotes, and their contribution is particularly noticeable among UK patients (Table 2). In analysis of UK iatrogenic growth hormone cases 35 of 1880 (1.9%) developed CJD. Of the 20 cases codon 129 genotyped, 11 were valine homozygotes (55%) with only 1 methionine homozygote. The appearance of this susceptible group may be simply that the contaminated donor shared the same codon 129 genotype and therefore those recipients who share the same genotype are most susceptible, however the low levels of iatrogenic cases suggest that susceptibility will be due to additional factors.

In the French studies heterozygotes were found to be significantly ($p=0.003$) less susceptible and had longer incubation periods than homozygotes, however studies of

129 genotype in cases in the United States and the UK could find no significant association between incubation periods and codon 129 genotype [57].

Table 2

Iatrogenic Patients	Number tested	Met / Met	Met / Val	Val / Val
UK	20	1	8	11
USA	9	5	2	2
FRANCE	53	33	7	13

Codon 129 genotype of patients in different countries who contracted CJD via growth hormone treatment

Increased knowledge of the transmissibility of CJD, improved surgical instrument decontamination, plus the availability of recombinant growth hormone and factor VIII products should eventually eliminate the risk of iatrogenic CJD. Concerns remain over the potential transmission of vCJD through blood and blood products, which have been justified considering the recent emergence of a case of vCJD in the recipient of a blood transfusion from a donor who later died of vCJD [75,76] (see vCJD section). The episode of vCJD transmission by blood transfusion remains a possibility and is not a proven causal connection; it is though a recognised risk. If transfusion was the cause of disease transmission it illustrates that blood can be infectious when the disease is at an undetected preclinical stage, and that there is an extremely long incubation period before the emergence of disease in the recipient. This connection was identified by means of collaboration between the National CJD Surveillance Unit and the UK national blood authorities known as the 'Transfusion Medicine Epidemiology Review'. As part of this study patients who receive blood

products and develop vCJD are investigated and transfused products are traced to donors.

1.6.3 Familial CJD, GSS, and FFI

Hereditary CJD, Gerstmann-Straussler-Scheinker Disease (GSS) and Fatal Familial Insomnia (FFI) are associated with mutations in the open reading frame of the prion protein gene (*PRNP*) and are inherited in an autosomal-dominant manner. Mutations can be point mutations where one amino acid is substituted for another. Insertions can also occur where 2-9 copies of a 24 base pair region encoding the octapeptide repeat sequence are added between codons 51-91 [77,78]. There exists a great deal of heterogeneity in disease phenotype in this group of inherited conditions as result of direct effects of the mutation coupled with additional effects of the codon 129 genotype. This combination is referred to as the disease associated haplotype of which more than a dozen have been characterised in hereditary CJD and one in FFI [78,79]. All hereditary CJD, FFI, GSS are transmissible. The mutation's effect is to cause a disruption in the conformational structure of the prion molecule, and it is this change in conformation which determines the disease phenotype. These changes in conformation can be visualised by western blot analysis following PK treatment. An analysis of the Asparagine (N) to Aspartate (D) substitution at codon 178 where the codon 129 genotype was valine homozygous (D178N-129V) differed in migration and glycosylation pattern to FFI mutation D178N-129M haplotype [80], these migration and glycosylation patterns are now referred to as type 1 and type 2 as mentioned earlier in classification of sporadic CJD. Different mutations and PrP^{Sc}

conformation therefore appear to be critical to disease phenotype, since one causes familial CJD and the other FFI.

Hereditary CJD accounts for 10 % of human prion disease and is associated with over 20 different combinations of mutation and codon 129 genotype. The most common haplotype in inherited CJD is caused by substitution of Lysine (K) for Glutamate (E) at codon 200 with Methionine homozygosity at codon 129 (E200K-129M). Due to the nature of inheritance clusters or kindreds occur, for this particular mutation large clusters have been discovered amongst Jews of Libyan origin and in Slovakia and Chile [81-84]. The onset of disease associated with this mutation is quite variable occurring between ages of 40-80 with a mean duration of 6 months. Clinical features resemble sporadic CJD associated with Methionine homozygosity at codon 129, with type 1 PrP^{Sc}. The same codon substitution associated with Valine at codon 129: E200K-129V is associated with a rapid progressive dementia, ataxia, and type 2 PrP^{Sc} similar to Valine codon 129 sporadic CJD cases.

Other well-characterised haplotypes include D178N-129, which was the first familial CJD case recorded in 1924, V210I-129M, V180I-129M, and M232R-129M haplotypes.

In addition a considerable number of PRNP haplotypes have been found in isolated cases of CJD, affecting single subjects which lack family history, therefore linkage analysis is unavailable and it is difficult to assess whether the mutation is the cause of the condition. These include I213M, G142S, Q160S, T188R, R208H, T188K,

T188A, E196K, V203I, E211Q, P238S [78]. This group of mutations exhibit a clinical disease phenotype identical to that of sporadic CJD MM1.

The remaining mutations involve either insertion or deletions within the octapeptide repeat region between codons 51-91. The insertion of between 1 and 9 (but not 3) extra octapeptide repeats is associated with hereditary CJD. More than 20 insertion mutations which differ in number and arrangement of repeats have been described which affect 20 families with 95 affected individuals [78]. Deletions of 1 octapeptide repeat sequence is a common mutation which occurs in 1-2.5% of the population and is not associated with the development of CJD [85]. However the deletion of 2 octapeptide repeat sequences is associated with CJD and has been characterised in 2 individuals [86,87].

FFI is the third most common type of hereditary CJD linked to D178N-129M haplotype. This mutation is also responsible for a form of familial CJD (D178N-129V) methionine homozygote at codon 129. Disease course in FFI is influenced by codon 129, as expected homozygotes have a shorter disease duration $12^{+/-}4$ months compared with heterozygotes $21^{+/-}15$ months [88]. Clinical signs include sleep, autonomic and motor function abnormalities. Insomnia, drowsiness, and apathy are symptoms along with autonomic, endocrine disturbance. The distinct feature of FFI is severe atrophy of the anterior ventral, mediodorsal and pulvinar thalamic nuclei accompanied by the loss of the majority of neurons and a significant increase in levels of astroglial cells [78,89]. PrP^{Sc} deposition is usually absent in FFI cases except in cases where there is a long disease duration, and when found is of type 2 [90].

1.6.4 Kuru

Kuru is a neurological condition historically endemic amongst the Fore linguistic tribes resident in the eastern highlands of New Guinea since 1941. Cannibalistic rituals were established as the cause of the disease transmission [91]. These cannibalistic rituals were abandoned by the late 1950s so halting disease exposure, however due to the long incubation periods associated with the disease, Kuru maintained its incidence for a further 40 years. Although Kuru was seen to share characteristic features with sheep scrapie, it was not until successful experimental transmission to apes that it was established as a form of TSE [92].

The disease duration ranges from between 4-24 months and is clinically characterised by uniform pan-cerebellar dysfunction. Homozygosity at codon 129 of the *PRNP* gene especially methionine homozygosity is linked to a younger age at onset and a shorter disease duration [93]. Characteristic neuropathological features include neuronal loss, myelin degeneration, astrocytic gliosis, microglial proliferation, spongiform change associated with the deposition of PrP, and the presence of amyloid PrP plaques [94].

The study of Kuru, an oral transmissible disease occurring in humans has important implications in the study of vCJD which is linked to consumption of BSE infected beef. The two diseases are phenotypically similar, and studies of Kuru can be used as essential background for the eventual elimination of vCJD.

1.6.5 Variant CJD

New variant Creutzfeldt-Jakob disease (vCJD) was described in 1996 as a novel transmissible spongiform encephalopathy (TSE)[58]. 10 cases were reported all with neurologically unique profiles, affecting relatively young people with a clinical course and disease duration atypical of sporadic CJD. Clinical features included psychiatric and sensory disturbance, dysaethesiae, foot pain, ataxia, and memory impairment, all patients developed progressive dementia. Myoclonus was also a feature later in the clinical course. In addition EEG features normally associated with sporadic CJD were absent. Neuropathologically vCJD cases showed spongiform change, neuronal loss, and PrP plaque formation. Spongiform change and astrocytosis were most evident in the thalamus and basal ganglia, but also present in the cerebrum and cerebellum. The most unusual feature was the presence of PrP plaques in cerebrum, cerebellum, thalamus and basal ganglia. The plaques were unique and resembled the florid-like plaques first found in the transmission of Icelandic scrapie in mice and recently reported in a subset of Japanese dura mater iatrogenic cases [95]. They have a dense glassy eosinophilic centre and pale periphery of radiating fibrils surrounded by a halo of spongiform change see Figure 7.

Figure 7

The cerebral cortex of a patient with vCJD. Arrow locates a plaque surrounded by a ring of spongiform change.

PrP deposition present in these plaque regions in the cerebral cortex, and cerebellum, can also be found in the thalamus, basal ganglia, and grey matter. Clinical course, neuropathology and young age of patients are all very distinct from those you would associate with sporadic CJD. Genetic analysis found all cases to be MM at codon 129 in the *PRNP* gene, this group would therefore appear to have increased susceptibility to infection with the BSE agent which only codes for methionine at the equivalent site to the 129 polymorphism and is thought to be responsible for vCJD in the human population. This is not to say that heterozygous or valine homozygote individuals are resistant to vCJD. It may be that these individuals could be affected after prolonged incubation periods. Certainly codon 129 genotype influences incubation periods in recipients of human growth hormone [74] as discussed earlier.

There is compelling evidence that vCJD results from the consumption of beef products infected with BSE. Transmission studies in mice suggest that the same strain of prions cause BSE and vCJD, both show similar incubation periods and neuropathology [38]. Molecular strain typing studies using western blotting techniques support this hypothesis [96], the vCJD prion is heavily diglycosylated with a fragment pattern distinct from those associated with other human TSEs. However, this evidence although compelling only indicates that BSE and vCJD are caused by the same strain of agent.

vCJD is transmitted by inoculation with, or dietary exposure to infected tissues, and in contrast to other human TSEs PrP^{Sc} can be detected in lymphoreticular tissues of patients with vCJD [97-99]. PrP^{Sc} was readily detected in spleen and lymph nodes of vCJD patients with the highest levels detected in tonsil. Lower levels could also be detected in the optic nerve, rectum, adrenal gland and in the thymus of 1 patient [97,98]. PrP^{Sc} deposition could not be found in the same peripheral tissues from other human prion diseases or controls. Tonsil and spleen samples from patients with vCJD have also been found to harbour infectivity which can be transmitted when bio-assayed in mice[100].

The process of neuroinvasion following oral exposure in humans is not clearly understood. PrP^{Sc} is known to accumulate and replicate in peripheral lymphoid tissues prior to neuroinvasion. Follicular dendritic cells are known to replicate and accumulate PrP^{Sc} in secondary lymphoid follicles in animal models [101]. Complement factors would appear to have an important role in modulation of prion replication since mice deficient in these factors were partially or fully protected from infection

with scrapie [102]. The sympathetic nervous system innervates the gastrointestinal tract, the spleen and lymph nodes and is therefore thought to be most relevant avenue for neuroinvasion. Experiments in mice comparing wild-type and mice over-expressing PrP^c found that the overexpression of PrP^c by neurons of the peripheral nervous system accelerated neuroinvasion [103]. Studies suggest that these nerves may not only be involved in prion transport but may also accumulate PrP^{Sc} in lymphoreticular tissues [104].

The role of the sympathetic nervous system as a bridge for CNS invasion is supported by two recent pieces of research. The first describes the presence of PrP^{Sc} in the celiac and stellate ganglia of the autonomic nervous system in patients with vCJD, deposition was not found in patients with sCJD [105]. The second illustrates how ablation of chemokine receptor CXCR5 causes the repositioning of follicular dendritic cells in germinal centres in secondary lymphoid follicles of mice so they are closer to sympathetic nerves. This was found to increase the efficacy of prion invasion [106]. These two studies support the involvement of the sympathetic nervous system in transmission of PrP^{Sc} from the periphery to the central nervous system, and support an oral route of infection for vCJD. However the exact mechanism of transport along nerve systems remains unclear, as does the interaction between immune cells and nerve endings.

The presence of detectable PrP^{Sc} in peripheral tissues may provide scope for the development of a preclinical screening test, and raises the question of the potential for its transmission by blood transfusion. This raises the possibility of a major

epidemic of vCJD occurring as a result of dietary exposure to BSE prions, and/or iatrogenic exposure to vCJD prions. Furthermore since little is known regarding incubation time and blood infectivity prior to the appearance of clinical symptoms, it is possible that infected blood donors may threaten the safety of blood transfusion. Indeed the circumstances surrounding the first possible case of transfusion-transmitted vCJD reported in December 2003 cause concern [76].

It has been difficult to predict the eventual number of individuals who will go on to develop vCJD since the variables which would enable a prediction as to the eventual size of the epidemic such as incubation period, genetic susceptibility and infectivity levels in food chain are not known. Due to uncertainties about incubation period mathematical modelling estimates that the vCJD outbreak could affect numbers ranging from less than a hundred to several hundred thousands [107,108]. A more recent estimation of epidemic sizes based on age-risk assessments gives the total number of cases to be 205 and the mean duration of incubation period to be 16.7 years assuming that all infections occurred between 1980 and 1989 and that the risk of developing the disease in exposed subjects decreases exponentially with age after age 15 [109]. A recent decline in the incidence of vCJD has lead to revised predictions for the eventual numbers of affected individuals. Indeed since a maximum of 28 cases in 2000, the incidence has declined considerably to 20 cases in 2001, 17 cases in 2002, and 16 cases up to 1st December 2003. A recently published update models projections of vCJD deaths in the UK by relating estimated exposure to BSE infected animals to vCJD case data. This estimates the future number of cases to be 40 with 95% prediction accuracy with an upper limit of 500 cases. The mean incubation

period was calculated at 12.6 years [110]. Integrating the data generated from the detection of 1 positive appendix in 8318 tissues tested in a recent study [111] the estimates increase to 100 cases with an upper limit of 2500. Since all individuals who have developed vCJD so far are codon 129 methionine homozygotes it may be that these are the most susceptible group and that the remaining types have been infected but incubate over a longer period prior to clinical presentation. It is known from epidemiological studies on Kuru that incubation periods can be as long as 40 years before the appearance of clinical disease signs. It is unlikely that epidemics will arise in these groups since their non-emergence is either due to a longer incubation period or reduced susceptibility.

The recent emergence of a possible case of transfusion related transmission [76] is cause for concern since it is possible that preclinical cases may harbour infectivity and thus a blood screening strategy is desirable to identify those blood products which contain vCJD infectivity so they can be removed from the blood supply. In 1996 an individual aged 62 years received a transfusion of 5 units of red blood cells. Of these products one had been donated by an individual of 24 years who went on to develop vCJD symptoms 3 years 4 months later and died in 2000 of pathologically confirmed vCJD. The recipient began to develop symptoms in late 2002 of depression and unsteady gait and died 13 months later. Due to the age of the patient and normal MRI scan the cause of death was recorded as dementia, although the post mortem reported features indicative of CJD and subsequent investigations confirmed the codon 129 genotype to be methionine homozygous and biochemically confirmed the presence of type 2B prion protein (vCJD) in the brain, and the presence of florid

plaque deposition. Data indicates that if transfusion was the cause of disease transmission that the blood of the donor was infectious 3 years before disease developed, and that there was an incubation period of 6.5 years before disease development in the recipient. This is cause for concern since it is known that of 135 vCJD cases 15 have donated blood from which 48 products have been transfused to recipients. Of this number 41 received red blood cell components a third of which were leucodepleted by filtering to contain <5million leucocytes per unit after the introduction in 1999 of leucodepletion of UK blood supply as a precautionary measure against vCJD transmission. 6 received plasma components, and one received platelets. Of these recipients none are known to have donated blood, 31 have died one of which is the vCJD case, and 17 remain alive.

Table 3

(From http://www.cjd.ed.ac.uk/)

*As at 4th March 2005

Summary of vCJD cases

Deaths	
Deaths from definite vCJD (confirmed):	106
Deaths from probable vCJD (without neuropathological confirmation):	42
Deaths from probable vCJD (neuropathological confirmation pending):	1
Number of deaths from definite or probable vCJD:	**149**
Alive	
Number of definite/probable vCJD cases still alive:	5
Total number of definite or probable vCJD cases (dead and alive):	**154**

There is growing evidence from studies of mouse scrapie that the initial TSE infection is carried by cells of the immune system to lymphoreticular tissues prior to neuroinvasion of the CNS [112-115]. sCJD can be transmitted by intracerebral inoculation of blood and buffy coat from clinically affected patients [116], and recent reports of the transfusion of natural scrapie and experimental BSE by buffy coats support this theory [117]. However despite this, transmission of sCJD by blood transfusion does not pose a significant clinical problem. Indeed it has been difficult

for transmission to occur via the intravenous route, much higher doses were required for successful transmission in studies of mouse adapted TSE [118].

Numerous animal studies have found low levels of infectivity detectable in circulating blood from rodents experimentally infected with CJD, BSE, and scrapie during incubation and clinical phases of disease [119]. Recent studies document the experimental transmission of BSE between sheep by blood transfusion [120], the detection of infectivity in the plasma of mice infected with mouse adapted BSE [121], and in the buffy coat of a prosimian model experimentally infected with BSE [122].

Infectivity had not been detected in blood from animals with naturally occurring scrapie until the recent report detailing the transmission of natural scrapie by blood transfusion [123]. Twenty-one sheep from a scrapie-free flock were transfused with blood either whole blood or buffy coat fractions taken from scrapie-infected animals at different time points. At the time the article was published 4 animals had developed clinical signs of scrapie receiving blood at 57, 69,77 and 100% of clinical phase of the disease in donor animals at the time at which blood was taken. Three received whole blood transfusions, 1 buffy coat transfusion. Recent figures from this study [117] now indicate 43% (9/21) of sheep transfused with blood from sheep with natural scrapie have developed the disease, incubation period ranges from 575-1138 days post transfusion. The blood donations were between 53-100% (clinical phase) of donor survival time. Of the 9 cases of transmission, 4 were whole blood transfusions developing between 575-1138 days post transfusion, and 5 were buffy coat transfusions developing 737-1101 days post transfusion.

Reports illustrating the transmission of CJD by human peripheral blood, for example transmission by buffy coat from sCJD patients to hamsters [116], must be assessed and balanced against large numbers of studies which have failed to find infectivity [124]. More importantly until the recent report of the possible transmission of vCJD by blood transfusion [76] no case of CJD resulting from the transmission of human blood or blood products had been identified through epidemiological investigations [125-128], and it remains a possibility that the individual may have developed vCJD by oral exposure to the BSE agent rather than the disease developing as a result of blood transfusion.

The risk of transmission by blood from patients with vCJD may be increased by the presence of PrP^{Sc} in the reticuloendothelial tissues [97,100,129]. Studies assessing the infectivity of vCJD blood provide conflicting results. Early studies discovered it was not possible to transmit vCJD to mice via intracranial inoculation of either buffy coat or plasma [100]. More recent studies have found the presence of infectivity in vCJD blood when transmitted to mice [130].

Considering current precautionary policies of universal leucodepletion and processes involved in plasma fractionation which attempt to reduce the risks, it is unlikely that transfusion based transmission is occurring at a high level [131]. The recent possible transmission of vCJD via blood transfusion has prompted the latest ban on donors who have received blood transfusions since 1st January 1980. The key uncertainty in attempting to evaluate the risk of vCJD transmission by blood transfusion and evaluate the efficacy of leucodepletion and plasma fractionation are the numbers of

blood donors who may currently be incubating the disease and the level and distribution of infectivity in the peripheral blood in these individuals. In the isolated case of transfusion related transmission reported in December 2003 the blood was donated 3 years before disease development in the donor illustrating that the donated blood if it was the cause of vCJD in the recipient was infectious many years before disease onset. There is therefore a need for a peripheral blood screening assay capable of identifying those currently in the preclinical phase of infection to allow estimation of the public health problem, deferral of high risk blood and organ donors, and the eventual clinical trials of potential prophylactic and/or therapeutic agents prior to the onset of clinical disease [132].

1.7 Development of assays for the diagnosis of TSEs

The common feature of all TSEs is the accumulation of PrP^{Sc} in the CNS and in some cases in the peripheral tissues. PrP^{Sc} is recognised as 'self' and so does not promote immunological responses and there are no detectable nucleic acids associated with PrP^{Sc}. Direct PCR based approaches for detection of a nucleic acid component are not applicable. PCR amplification of nucleic acid antibody linker molecules have been used in diagnostic assays but suffer from background problems. Pre-mortem diagnosis with the exception of a clinical diagnosis is difficult since PrP^{Sc} is concentrated in the CNS with very low concentrations detectable in the peripheral tissues. As a consequence, the detection of PrP^{Sc} as a marker for disease is best suited to post-mortem neuropathological examination, which at present represents the gold standard for the diagnosis of TSEs. Post mortem tests include immunohistochemistry, ELISA, western blotting, and mouse bioassay.

Immunohistochemical and biochemical approaches exploit the protease sensitivity of PrP^{c} to identify PrP^{Sc} whose conformationally altered structure is largely resistant to protease action. An anti-PrP antibody used in either an ELISA or western blotting assay is able to detect the disease-associated protein. Post-mortem testing using these methods are employed in cattle slaughterhouse tests to ensure no BSE infected beef enters the human food chain. There are currently 5 tests available for this purpose that have undergone evaluation by the European Union. The Enfer TSE kit 2.0 is an ELISA assay, which detects PrP^{Sc} in PK digested brain homogenate with a polyclonal antibody coupled with an enzyme conjugated secondary antibody. The signal is detected by chemiluminescence. The Prionics–Check LIA uses an identical approach to the Enfer test except that it uses monoclonal antibodies one of which is enzyme conjugated for the detection of PrP^{Sc} after PK treatment. The signal is detected by chemiluminescence. Another ELISA based assay system is the Biorad Platelia which again relies upon PK digestion and is followed by a precipitation and centrifugation step. PrP^{Sc} is detected in a sandwich ELISA using a colour-converting enzyme conjugated detection antibody. Other assays include the Prionics-Check Western where the PK digested brain homogenate is separated by SDS-PAGE and transferred to PVDF membrane. Separated PrP^{Sc} is detected using a specific antibody and an alkaline phosphatase-conjugated secondary antibody. Finally the InPro Conformation-Dependent Immunoassay relies upon protease digestion of PrP^{c} but identifies the presence of PrP^{Sc} based upon monoclonal antibody binding to an epitope that is hidden in PrP^{Sc} but exposed in PrP^{c}. When denatured more antibody will bind to a sample containing PrP^{Sc} due to the exposure of the hidden epitope.

Protease treated brain homogenate is treated with phosphotungstate which selectively precipitates PrP^{Sc}. After centrifugation the resuspended sample is divided into two, 1 half is denatured at high temperature the other half (native) sample is left untreated. Samples are assayed on an ELISA plate precoated with an anti-PrP monoclonal antibody. A europium conjugated detection antibody is used to bind any PrP present, and the europium signal is enhanced and read on a time-resolved fluorometer. If PrP^{Sc} is present then the ratio of the signal of the denatured sample divided by the native sample will be greater than that of a control uninfected sample.

Currently the most widely used tests in Europe are the Prionics-Check western and the Platelia test. The BioRad test is capable of detecting PrP^{Sc} up to 3 months prior to the emergence of clinical symptoms.

In most TSEs the deposition of PrP^{Sc} is confined to the CNS, in scrapie in sheep and goats and vCJD in humans it can also be readily detected in the peripheral nervous system and in the lymphoid system in tissues such as the spleen, tonsil and appendix including numerous other tissues (Table 4).

Table 4

Peripheral Nervous System	vCJD	sCJD
Olfactory nerve, cribiform plate	NT	√
Optic nerve, neural retina	√	√
Trigeminal nerve	√	√
Alveolar nerve	-	-
Sciatic nerve	-	NT
Celiac and stellate ganglion	√	-
Lymphoreticular System		
Tonsil	√	-
Gut associated lymphoid tissue	√	-
Spleen	√	√
Appendix	√	-
Lymph nodes (mesenteric and cervical)	√	-
Eye and Oral cavity		
Cornea, iris, lens, vitreous body, choroids, sclera	-	-
Neural retina and optic nerve	√	√
Dental pulp, gingival, alveolar nerve, salivary gland, tongue	-	-
Organs / tissues		
Heart, liver, lung, kidney	-	-
Thymus, adrenal gland	√	-

Tissue distribution of PrP^{Sc} outside the CNS in patients with vCJD and sCJD, data sourced from [98,99,105,133,134]. Presence (√); absence (-), not tested (NT)

This has allowed the tests described above to be used in the preclinical diagnosis of scrapie. The preclinical detection of vCJD would be possible as illustrated by a recent retrospective study into tonsils and appendix in the UK where 1 vCJD case was identified [111]. However tests on human peripheral tissues are far too invasive to be routine, and a more easily accessible fluid such as blood or urine would be the sample of choice. The level of PrP^{Sc} in blood or urine is likely to be extremely low, if present at all, so preclinical screening tests based upon the detection of PrP^{Sc} would need to be extremely sensitive. Table 5 summarises the sensitivity of several

promising assay platforms which may have application as screening tests in the future.

Table 5

Assay technique	PrP^{Sc} preparation	Detection limit	Sensitivity ($ID_{50}mL^{-1}$)
Prionics check western	BSE brain homogenate	10^{0}-10^{1}	100 000-1000 000
Enfer TSE kit	BSE brain homogenate	$10^{-1.5}$	30 000
CDI DELFIA	vCJD brain homogenate	10^{-5}-10^{-6}	
	Hamster scrapie brain homogenate	10^{3} ID_{50} mL^{-1}	1 000
Wallac DELFIA	BSE brain homogenate	10^{0}	1000 000
CEA ELISA	BSE brain homogenate	$10^{-2.5}$	3 000
Capillary Electrophoresis	Scrapie in sheep buffy coat	3-135pg PrP^{Sc}	100-2500

Sensitivity of assays for detection of PrP^{Sc} adapted from MacGregor [16], and Ironside [135].

Recent developments which may allow the sensitive detection of PrP^{Sc} include the use of reagents which selectively bind and allow precipitation and purification of PrP^{Sc} including NaPTA [21], plasminogen [136], and protocadherin. A reagent that would transform TSE diagnostics is the availability of a PrP^{Sc} specific monoclonal antibody that would allow the specific detection of the disease-associated protein without the need for proteases that may also remove some protease sensitive disease associated proteins. There have been some recent developments including an anti-nucleic acid antibody which appears to have specificity for PrP^{Sc} alone [37], similarly a monoclonal antibody to a conserved tyrosine-tyrosine-arganine tri-peptide motif which is exposed in PrP^{Sc} but not in PrP^{c} was able to precipitate PrP from TSE-infected but not uninfected brain homogenates [137]. A different approach has been an in-vitro

amplification technique which allows rapid conversion of an excess of PrP^{c} to PrP^{Sc} in the presence of a small quantity of PrP^{Sc} [27]. This may allow the detection of minute quantities of PrP^{Sc} present in tissues and biological fluids. Recent studies using this technique have found that the transformation in vitro also requires specific RNA molecules, suggesting that host-encoded catalytic RNA molecules may play a role in the pathogenesis of prion disease [28]. Another approach is the use of capillary electrophoresis for the detection of PrP^{Sc} [138]. A fluorescent-labelled peptide of part of the amino-acid sequence of PrP is incubated with an antibody specific to this peptide at a concentration where 50% of the peptide is bound by the antibody. The mixture is then analysed by capillary electrophoresis and detected using laser-induced fluorescence intensity to provide information on the ratio of bound to unbound peptide. The addition of a sample containing PrP (any form of PrP) would compete with the peptide for antibody binding thereby disturbing the ratio. Published data claims that this sensitive assay is capable of detecting PrP^{Sc} in the blood of scrapie infected sheep and goats however these results have not been reproducible for the detection of PrP^{Sc} in the blood of chimps experimentally infected with CJD or CJD in humans [139]. There are also some highly technical spectroscopy approaches for example the use of Fourier transform infrared spectroscopy (FT-IR) as a test for scrapie and BSE in cattle has been described [140,141]. Analysis of blood serum by FT-IR and by artificial neural networks or multivariable pattern recognition analysis allows the sensitive and specific discrimination between healthy and infected animals. A method of fluorescent correlation spectroscopy has also been described which has some diagnostic sensitivity for the detection of PrP^{Sc} in the cerebrospinal fluid (CSF) of patients with CJD [142].

Since PrP^{Sc} is present at extremely low concentrations outside the CNS and is only likely to be present in detectable concentrations relatively late on in disease pathogenesis, alternative disease associated markers have been explored for the preclinical diagnosis of CJD.

One such marker is Erythroid differentiation-related factor (EDRF) whose expression was found to be down-regulated in an analysis of 10,000 RNA transcripts from spleens of scrapie infected mice. Down regulation was also found to occur in scrapie in sheep and BSE infected cattle when compared to healthy controls [143]. The lack of disease controls in this study is a worry for the disease specificity, but if specific, EDRF transcripts can be measured in blood by northern blot and would be a suitable screening strategy for the detection of TSEs to ensure the safety of human blood products within the transfusion services.

Another such marker is the human leukocyte antigen (HLA) class II DQ7 allele which was found to have a reduced expression in patients with vCJD but not in patients with sCJD or in organ donor controls. HLA and DRB tissue typing of 50 patients with vCJD found 6 (12%) expressed DQ7, the same allele was expressed by 12/26 (46.2%) of sCJD patients and by 70/197 (35.5%) of organ donor cadavers. The reduction of expression in patients with vCJD was found to be highly statistically significant ($p=0.001$). These results for Caucasian individuals suggest a protective effect of DQ7 and HLA typing may be useful as one of a number of tests for blood to identify risk groups given the association of HLA with other infectious diseases such as Human Immunodeficiency Virus (HIV) and hepatitis B.

Using a similar approach the presence of cellular nucleic acids circulating in the plasma of BSE infected cattle have been reported to correlate with disease [144]. Another promising technique uses a sensitive electrocardiogram system used in heart monitoring to measure a unique heart rate signature present in the early stages of disease. Tests on 150 cows experimentally infected with BSE found increased levels of respiratory sinus arrhythmia in 2 animals that died 8 months later, and suggest that this testing may be able to identify individuals with preclinical vCJD [145].

There are therefore several promising surrogate markers for the detection of TSEs which may contribute towards the development of a preclinical screening test for TSEs in humans and in animals. At present the clinical diagnosis of both sCJD and vCJD are based upon a series of diagnostic techniques with varying sensitivity and specificity along with clinical observations. It is likely that sensitive and specific preclinical diagnosis will be based upon several diagnostic criteria.

1.8 AIMS

This thesis seeks to address the following interrelated topics:

1. Establish and apply analytical techniques of dissociation enhanced lanthanide fluorescence immunoassay (DELFIA), and flow cytometry to assess the qualitative and quantitative distribution of prion protein (PrP) in the peripheral blood of patients with vCJD and sCJD. Do there appear to be differences in the level of expression of PrP in blood components, and does flow cytometric analysis highlight differences in the cell surface expression of PrP?

2. Use available techniques including the use of chaotropic salts and proteinase K (PK), to assess the possibility of detecting the abnormal prion isoform PrP^{Sc}. Apply to peripheral blood and tissues in cases of vCJD using patients with sporadic CJD (sCJD) and other neurological conditions along with anonymous normal volunteers as controls in these studies.

3. Develop a preclinical diagnostic test for vCJD that may also have application in verifying the safety of donated blood and blood products.

4. Apply ADR spectroscopic techniques for classification of clinical CJD and control blood samples.

Chapter 2: Materials & Methods

2.1 Ethics approval and patient consent

Before commencement, the study received approval from the Scottish Multi-Centre Research Ethics Committee and the Local Research Ethics Committees

2.2 Collection of Blood Samples

Anonymised whole blood samples from 30 healthy adult apheresis platelet donors were collected by the Scottish National Blood Transfusion Service Edinburgh and stored for 24 hours at 4° C before separation in order to mimic the conditions of collection of samples from CJD patients around the country. Blood from CJD patients and neurological controls was leftover from samples obtained by the National CJD Surveillance Unit for genetic analysis. Samples were not obtained solely for this study due to the difficulties of obtaining blood from these patients. Whole blood samples from 10 vCJD patients, 10 sCJD patients and 8 neurological controls were used for DELFIA studies. Informed consent was obtained from patients and donors for experimentation and ethics approval for the study was obtained as described in section 2.1. All vCJD and sCJD cases had a probable or definitive diagnosis based upon internationally established criteria [146,147]. Neurological controls were samples referred to the CJD Surveillance Unit from patients that subsequently did not meet criteria for a diagnosis of definite or probable CJD. These 8 patients were subsequently diagnosed with neurological disorders distinct from CJD including Alzheimer's disease (2), paraneoplastic syndrome (2),

mitochondrial disease (1), Lewy body dementia (1), non-organic depression (1) and central pontine myelinolysis (1).

2.3 Time-resolved fluoroimmunoassay for Prion Protein

The highly sensitive method of time-resolved dissociation-enhanced fluoroimmunoassay (DELFIA®) was employed for the detection of PrP by sandwich immunoassay. This method was carried out based upon a previously described protocol [9]. The DELFIA employed monoclonal antibody FH11 [148] (produced at IAH Compton against ovine recombinant PrP, originator Dr C.R Birkett) as capture antibody, and monoclonal antibody 3F4 [149] (against hamster PrP and obtained from TSE Resource centre, IAH, Compton, UK, originator Dr Richard Kascsak) conjugated to europium^{3+} as signal antibody. Eu-labelling reagent and DELFIA assay buffer, wash concentrate, and enhancement solutions were obtained from PerkinElmer Life Sciences, Hounslow, UK.

The assay was calibrated using an expired platelet concentrate which had been calibrated with a recombinant human soluble PrP [150] (Catalogue number 03-041 Prionics, Switzerland). The recombinant human prion protein gene (*PRNP*) was amplified from genomic DNA, expressed and purified by Prionics following previously published methods [151]; its sequence constitutes amino-acid residues 23-230 and has methionine at position 129.

Initially europium labelled 3F4 monoclonal antibody and FH11 coated plates were obtained from PerkinElmer Life Sciences, UK. Test samples or calibrators diluted in assay buffer were applied 200 µL / well to 96 well microplates, (Immulon-4 HBX

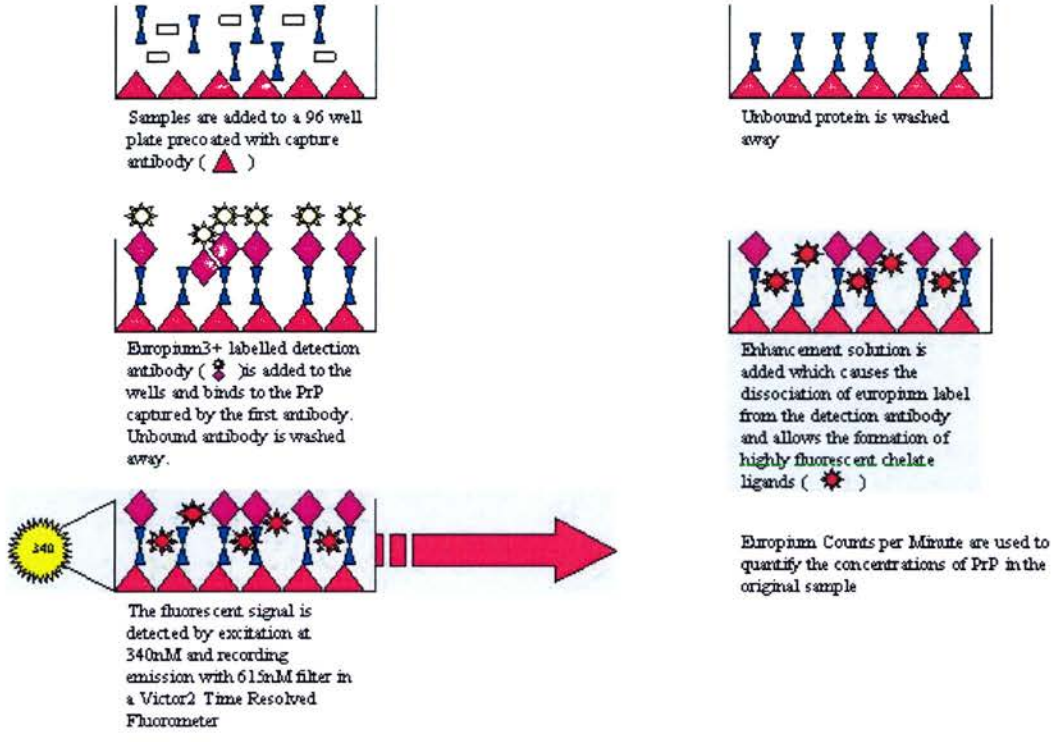
Thermolabsystems, Basingstoke, UK) pre-coated with FH11 and incubated for 1hour on a shaker (IKA labortechnik, Germany).

The wells were then washed four times with DELFIA wash buffer using a DELFIA® Platewash (PerkinElmer Life Sciences, UK) and charged with 200 µL / well of the 3F4-europium^{3+} conjugate at a dilution previously determined by titration against the platelet standard reagent. After incubation for a further 1hour on a shaker the wells were washed six times with DELFIA wash buffer after which 200 µL of enhancement solution was added to each well and the plate incubated on a shaker for 5min. This methodology is illustrated in Figure 8.

The acid-detergent-chelate enhancement solution liberates europium^{3+} from its chelation with the 3F4 antibody by low pH and allows the formation of a highly fluorescent chelate with ligands present in the enhancement solution. The resulting fluorescent signal was then measured with a Victor2 time-resolved fluorometer (PerkinElmer Life Sciences, UK).

Figure 8

Dissociation-enhanced lanthanide fluoroimmunoassay



For the quantification of PrP^c data was analysed using WorkOut software version 1.5 provided by PerkinElmer Lifescience. The concentration in Units/mL of five dilutions of expired platelet concentrate titrated against human recombinant PrP^c were used to calibrate the assay. 1 Unit was found to be equivalent to 26 picograms/mL. The software was programmed to subtract blanks from duplicate wells and to calculate duplicate means and express these in terms of a concentration in Units/mL.

2.3.1 Preparation of FH11 coated plates

Immulon-4 HBX 96 well microtitre plates were incubated overnight at room temperature (RT) with 200 µL/well 5 µG/mL FH11 in bicarbonate-carbonate coating buffer (Sigma Aldrich, UK) pH 9.6. Plates were washed 4 times with DELFIA wash buffer and blocked with assay buffer + 1 % bovine serum albumin (BSA) (Sigma Aldrich, UK) for 1 hour on a shaker at RT, then washed 4 times once again with DELFIA was buffer prior to loading of samples.

2.3.2 Preparation of Europium3+ conjugated antibody

Monoclonal antibodies used for detection were $europium^{3+}$ labelled using Eu-labelling kit 1244-302 from PerkinElmer Life Sciences, UK.

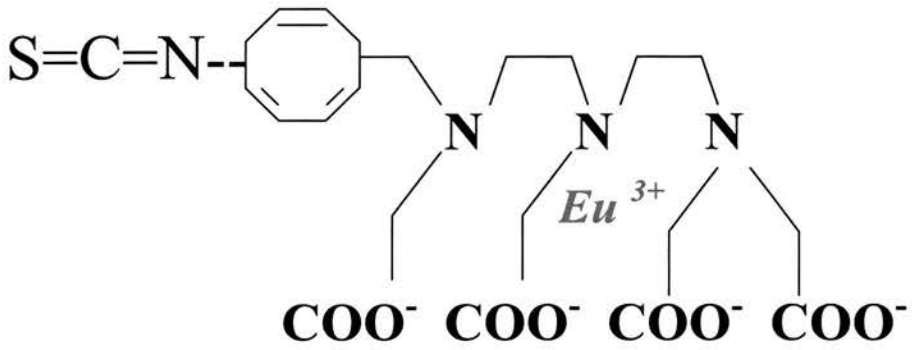
1mG of antibody whose concentration was determined by measurement of the absorbance at 280nM divided by the extinction coefficient for IgG (1.36) was added

to a Microcon-30 concentrator (Millipore (U.K.) Ltd, Watford, UK) and centrifuged at 10,000g. The eluate was discarded and the retentate washed 4 times with 400 μL labelling buffer (50mM $NaHCO_3$ pH 8.5). The washing step was essential to removed the phospho-buffered saline (PBS) and replace with carbonate buffer so as to ensure a suitable pH and to remove any basic charges from the buffer to reduce competition for the amine groups to which the europium binds.

The absorbance of each eluate obtained following centrifugation was measured using a spectrophotometer at a wavelength of 280 nM as an indicator for the presence of protein in order to monitor the retention of antibody in the microcon filter. The presence of protein in solution can be determined by the presence of aromatic amino-acid residues tyrosine and tryptophan which exhibit an absorbance maximum at 280 nM.

After the final wash step the retentate was diluted in 450 μL labelling buffer and added to 0.2mG Eu-labelling reagent n-1(p-isothiocyanatophenyl)-diethylene-triamine N_1,N_2,N_3-tetraacetate (Figure 9); (DTTA) chelated with europium and incubated overnight at + 4°C.

Figure 9



Chemical structure of the Eu-labelling reagent, N^1(p-isothiocyanatobenzyl)-diethylene-triamine- N^1, N^2, N^3-tetraacetic acid chelated with Eu^{3+}

The labelling reagent DTTA forms a stable complex with Eu^{3+} and the isothiocyanate group reacts with the primary aliphatic amino group on the protein at alkaline pH to form a stable covalent thiourea bond. The high water solubility and the stability of the chelate, in addition to the mild coupling conditions of the isothiocyanate reaction enable effective protein labelling.

This volume was then transferred to a Microcon-30 concentrator and centrifuged as before, the eluate was discarded. The retentate was washed 4 times using elution buffer (50mM Tris-HCL + 0.9% NaCl, 0.1% NaN_3; pH 7.8). The retentate was then diluted with 200 µL elution buffer and transferred to an antibody storage vial. The Microcon membrane was washed a further 4 times with 200µL elution buffer and each wash volume transferred to the antibody vial to give the final concentration of labelled antibody as 1 mG/mL. The labelled antibody was then filtered through a

Millex-GV4 (Millipore UK, catalogue number SLGVR04NL) to remove suspended particles and antibody aggregates, and BSA as a stabiliser added to a final concentration of 0.1 %.

In order to establish the labelling yield or the number of Eu^{3+} molecules bound to each IgG molecule, firstly the Europium content of the labelled antibody was calculated by comparison with a europium standard supplied with the labelling kit. The labelled antibody was diluted 1:100 000 in enhancement solution and applied twice in duplicate to a microtitre plate 200 µL/well. The europium standard was diluted to a final concentration of 1 nmol/L in enhancement solution and added to the plate as above. The europium content was calculated using the following equation:

$$Eu^{3+}\ (\mu mol/L) = Eu\text{-}counts \times dilution\ factor / 1000\ X\ counts\ of\ 1nmol/L\ Eu^{3+}$$

The protein content of the labelled antibody was then determined by measuring its absorbance at 280 nM and correcting for the molar absorptivity of reacted labelling reagent whose absorbance at 280 nM is 8000, 1 µMol gives an absorbance of 0.008. This value was then divided by the extinction coefficient for IgG 1.36 and multiplied by 100.

$$Protein\ (mG/mL) = [Abs\ 280nM - 0.008\ X\ Eu^{3+}\ (\mu mol/L) / 1.36] * 100$$

This value was then converted to µMol/L by multiplying by 1000000 and dividing by 160 000 g/L.

The yield was then calculated by dividing the europium content by the protein content as follows:

Yield (Eu^{3+}/IgG) = Eu^{3+} (μmol/L) / Protein (μmol/L)

2.3.3 Titration of expired platelet concentrate against human recombinant PrP^c

Recombinant human PrP^c (Prionics, Switzerland) was diluted in doubling dilutions in a range of 0.78 – 50 nG/mL and applied in duplicate 200 μL/well to a microtitre plate precoated with 5 μG/mL monoclonal antibody FH11. The assay was calibrated using the expired platelet concentrate at 5 fold dilutions from 1:5 –1:3125 assayed in duplicate 200 μL/well to provide a standard curve.

The calculations for each dilution of recombinant PrP^c in units based upon an earlier calibration where 1 unit = 200 pG/mL was found to be inaccurate and did not reflect the known concentration of the recombinant material. The concentration of platelet concentrate was calculated and 1 unit was found to be equivalent to 26 pG/mL.

2.4 Conformation dependent Immunoassay

This approach for the detection of abnormally folded PrP was adapted from methods developed by Safar et al [21]. The detection of PrP by MAb 3F4 in native and denatured portions of the same sample allows detection of abnormally folded PrP based upon the increased exposure of the 3F4 epitope on denaturation. The 3F4 epitope (αα 109-112 of PrP) is less accessible in abnormally folded PrP, but clearly

accessible in PrP^c. A signal increase in a denatured sample compared with a native aliquot of the same sample when assayed by DELFIA signifies the presence of an abnormally folded PrP.

50-100 μL of sample to be analysed was split into 2 aliquots of an equal volume in 0.5mL Eppendorf safe-lock tubes. The aliquots were labelled as either native or denatured, 8 Molar (M) guanidine hydrochloride (GdnHCL) (Sigma- Aldrich, UK) was added to the denatured aliquot to double its volume giving a final concentration of 4M. The sample was then heated to 80°C in a heat block for 6 min. The native aliquot remained untreated until diluted to 0.65 – 1.0 mL final volume with distilled water supplemented with protease inhibitors 5 mM PMSF + aprotinin and leupeptin (Sigma Aldrich, UK) at 4 μg/mL each + 0.308M GdnHCL. GdnHCL was added to this buffer so that it was present at the same concentration in both native and denatured samples. Denatured samples were brought to 0.65 – 1.0 mL with the same buffer without the GdnHCL. Samples were then applied in duplicate or triplicate 200 μL per well on a 96 well microtitre plate precoated with 5 μG/mL MAb FH11 or an alternative capture Ab. The plate was then incubated for 2 hours on a shaker, washed 4 times with DELFIA wash buffer, before blocking with 200 μL per well TBS (pH 7.8) containing 0.5 % weight per volume (w/v) BSA and 6 % w/v Sorbitol (Sigma Aldrich, UK) for 2 hours on a shaker following methods described previously by Bellon [152]. The plate was then washed 4 times and incubated with 200 μL per well Eu-labelled 3F4 (1/15000 dilution batch 29/10/03) for 2 hours at RT on a shaker. The plate was then washed and the signal enhanced and read following the standard DELFIA protocol as described in section 2.3.

2.4.1 Modified CDI including Sodium Phosphotungstic acid concentration and PK treatment.

The CDI protocol can be further adapted following methods of Safar et al [21] to incorporate the use of sodium phosphotungtic acid (NaPTA) (Sigma-Aldrich, UK) to enhance the detection sensitivity for PrP^{Sc}.

Samples (900 µL) to be analysed by CDI were brought to 1 % sarkosyl (a detergent) pH 7.4 by the addition of 100 µL of stock solution in a 1.5 mL screw-top tube (10 % w/v in PBS pH 7.4), vortexed, spun briefly to remove any liquid from the lid, and incubated at 37°C for 15 minutes.

2 µL of 25 Units/µL benzonase (Sigma Aldrich, UK) final concentration 50 Units was added to the tube along with 5 µL of 0.2M $MgCl_2$ (Sigma Aldrich, UK) to a final concentration of 1 mM. Samples were vortexed and incubated for a further 30 minutes at 37°C in a heat block.

81 µL of a pre-warmed (37°C) stock solution containing 4 % NaPTA and 170 mM $MgCl_2$ pH 7.4 was added to 1 mL samples to give a final concentration of 0.32 % NaPTA and 12.8 mM $MgCl_2$. Samples were vortexed and incubated overnight at 37°C in a waterbath.

Samples were then spun at 14,000g for 30 minutes in an Eppendorf benchtop 5417R centrifuge and the supernatant removed. Pellets obtained should be grey/brown in appearance and a white precipitate of magnesium salts should not be present. Pellets

were resuspended by pipette action in 50 µL distilled H_2O supplemented with 5 mM PMSF and aprotinin and leupeptin at 4 µg/mL each, this aliquot was split and sample processing continued following the standard CDI protocol described in section 2.4.

In order to increase sensitivity, prior to or following the addition of NaPTA, 12.58 µL 20 mG/mL PK (VWR, Poole, UK), final concentration 250 µG/mL was added and samples incubated for 1 hour at 37°C after which 10.2 µL 100mM pefabloc (Merck UK) was added to give a final concentration of 1 mM.

2.4.2 Modified CDI to incorporate NaCl precipitation.

Methods are exactly as those described in 2.4.1 apart from the following deviations: Following incubation of samples with benzonase and $MgCl_2$, 500 µL 30 % NaCL in PBS pH 7.4 (w/v) was added to each sample and samples were incubated on ice for 10 minutes with occasional vortexing. Samples were then centrifuged as described in 2.4.1, however, rather than resuspending the pellet immediately it was washed with 25 mM Tris-HCL pH 8.8 plus 0.05 % sarkosyl and spun as in 2.4.1. The pellet was then resuspended and assayed by CDI following methods described in section 2.4.1.

2.5 Flow cytometry

Flow cytometry analysis was performed by Dr Robin Barclay who kindly allowed his data to be presented in this thesis.

2.5.1 *Blood Samples*

Samples were processed by flow cytometry immediately on arrival at the National CJD Surveillance Unit, and subsequently assigned to clinical groups when diagnoses were made. An aliquot (0.4 mL) of whole blood from each sample was made available for flow cytometry studies. Any samples that did not fall into the diagnostic categories were not included in the analysis of results. Blood samples were also obtained from healthy adults and, because clinical samples were at least 24 hours old before arriving in the laboratory, these healthy adult control samples were processed by flow cytometry on the day following sampling.

2.5.2 *Pretreatment with proteinase K*

0.2 mL of the sample was set aside for staining (untreated). The remaining 0.2 mL was washed once in 2.5 mL of cold Cell Wash (Becton Dickinson), spun down and resuspended in 0.5 mL of proteinase K (PK) (Sigma Aldrich UK) at 1 mG/mL in Hank's balanced salt solution with calcium and magnesium (Sigma Aldrich UK) and left at room temperature for 30 minutes (the PK concentration for complete removal of cellular prion protein from healthy human blood cells under these conditions was determined by preliminary titration). The PK treated sample was washed four times in 2.5 mL of cold Cell Wash and the resultant cell pellet was divided equally between two 12 x 75 mm Falcon tubes (Becton Dickinson) for flow cytometry staining.

2.5.3 *Flow cytometry staining and analysis*

This was carried out essentially as previously described [6,10] except that a combination of MAbs 3F4 and 4F2 was used on the hypothesis that while MAb 4F2 clearly identifies PrP on human red cells, MAb 3F4 does not bind [29]. Therefore the 4F2 epitope on any PrP^{Sc} may be more susceptible to PK cleavage than the 3F4 epitope. These MAbs (5 μL each at 0.5 mG/mL) were added simultaneously to 100 μL of whole blood or PK treated washed blood with 100 μL of Cell Wash. Second samples of whole blood and PK treated blood received 100 μL of Cell Wash without anti-prion MAbs as unstained (negative) control. Following incubation and washing, anti-mouse Ig (human absorbed FITC-conjugated goat anti-mouse Ig F(ab')2, BioSource) at 1/500 in Cell Wash (100 μL) was added to all tubes. Following incubation and washing a sample (10 μL) was removed from each tube for red cell studies, and 5 μL of PerCP-conjugated anti-CD45 MAb and 5 μL of PE-conjugated anti-CD41 MAb (Becton Dickinson) was added to each whole blood tube. To each red cell study sample was added 5 μL of PE-conjugated anti-glycophorin A MAb (Serotec, Oxford, UK). Samples were incubated a further 30 minutes in the dark at room temperature. The red cell samples were washed twice with 2.5 mL of Cell Wash and resuspended in 0.5 mL of Cell Fix (Becton Dickinson). The whole blood samples were resuspended in 2.5 mL of lysing solution (Becton Dickinson) to remove red cells followed by 2 washes in 2.5 mL of Cell Wash and resuspended in 0.6 mL of Cell Fix. Fixed stained samples were kept overnight at 4°C in the dark before analysing by 3-colour flow cytometry as previously described [10]. Samples from lysed preparations were collected on linear forward/side scatter axes for leucocyte studies, and a second set were collected on logarithmic forward/side scatter

axes for platelet studies. Red cells were collected on linear forward/side scatter axes. A total of 20,000 events in appropriate forward/side scatter gates were collected to listmode files for each analysis. Analysis was conducted using FCS Express (DeNovoSoftware) on a PC. Samples were gated by appropriate forward/side scatter patterns and additionally by CD45 expression (different leucocyte populations); CD41 expression (platelets); or glycophorin A expression (red cells). Other markers of leucocyte populations (e.g. CD14) were susceptible to PK digestion and were not used. Histograms were made of channel-1 (FITC) expression for negative controls (no anti-PrP MAbs) and overlaid with histograms for channel-1 for anti-PrP (primary anti-PrP MAbs) for each cell class: single peaks were seen in each case and their median fluorescence intensities determined by setting appropriate markers on the histograms. Net median fluorescence intensity for anti-PrP staining was obtained by subtracting the negative control (no anti-PrP MAbs) value [10].

2.6 Total Protein measurements

Levels of PrP were expressed in Units per mL total protein for whole blood and all separated components to normalise data by compensating for the effects of separation protocol upon cellular particulate recovery in components and to ensure that range differences in cell number did not contribute to differences in PrP^c levels between groups. Although studies on full blood counts of clinical patient samples and controls did not show any abnormalities, full blood count data could not be generated from frozen stored clinical and control samples used in this study, therefore normalisation by total protein was essential for all components with the exception of whole blood.

Measurements of total protein levels were carried out using a Biorad Bradford protein assay (500-0002 Biorad Hemel Hempstead, Hertfordshire UK). The microtitre plate format was performed following the manufacturers instructions.

Briefly test and / or standard samples were added to 96 well microtitre plates in duplicate 5 μL/well. 25 μL/well of reagent A was then added to all wells followed by 200 μL/well of reagent B. The assay plate was incubated for 15 minutes on a shaker and read at a wavelength of 655 nM in a spectrophotometer (Biorad Benchmark, UK). The standard linear curve equation of the standard reagent allows calculation of the total protein levels in samples assayed.

2.7 Blood component separation protocol

Citrate anticoagulant blood samples taken from referred cases of suspected CJD or from controls were stored at + 4°C until separated into the component parts for storage at -80°C. All tubes were labelled with white laminating labels containing the Unit Associated Number and the suffix for the blood fraction (**B**=Whole Blood, **BC**=Buffy Coat, **P**=Platelet Poor Plasma, **PT**=Platelets, **R**=Red Blood Cells). Figure 10 provides a chart illustration of the separation process.

This method describes the separation of blood from one 9mL collection tube.
1.5 mL of whole blood was transferred to a sterile 2 mL tube (labelled 'B'). Then the remaining blood (~7.5 mL) from the vacutainer was transferred to labelled, sterile 15 mL Falcon for centrifugation in the sealed buckets of the Sigma 4-15C centrifuge (Sigma Aldrich, UK) and spun at 450g/ 1455 rpm for 10 minutes.

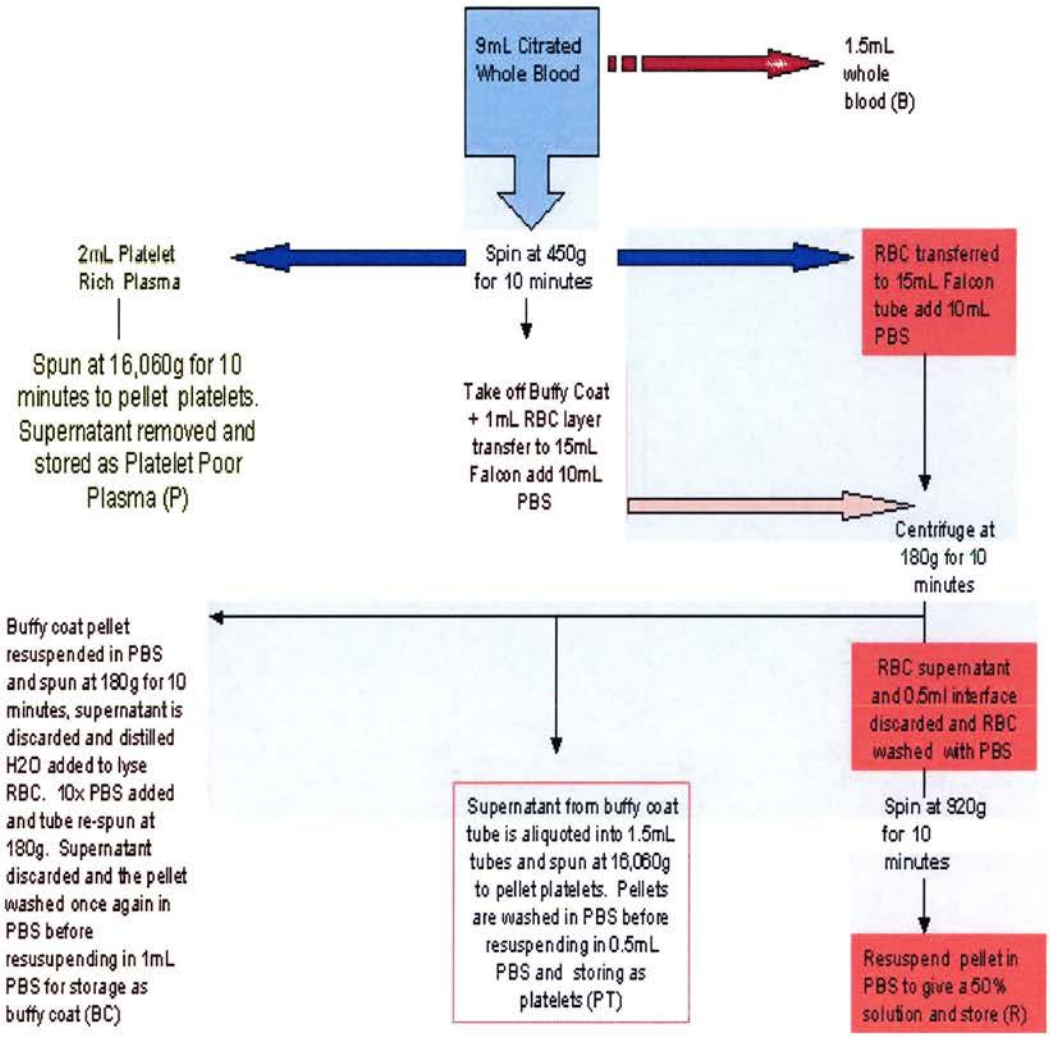
The top two-thirds (~2 mL) of platelet-rich plasma were transferred into labelled 1.5 mL conical tubes. The Buffy Coat including ~1 mL of the red cell layer (~2 mL in total with contaminating plasma, platelets, and red blood cells) was transferred to a labelled 15 mL Falcon. All but the bottom 0.5 mL of red cells were transferred into a labelled 15 mL Falcon. The Buffy Coat and Red Cell tubes were filled up to the 10 mL mark with PBS and spun at 180g/ 920 rpm for 10 minutes. During this spin step the tubes containing platelet rich plasma were spun in the Heraeus Biofuge at 16,060g for 10 minutes to pellet the platelets. After this spin the supernatant was transferred to 2 mL storage tubes and labelled as Platelet Poor Plasma (P), and the tubes containing the platelet pellets were discarded. The supernatant and the top 0.5 mL of the interface from the RBC tube was discarded into NaOH with a pastette and PBS was added up to the 14 mL mark. The tube was spun at 180g / 920 rpm for 10 minutes and the supernatant discarded. The RBC pellet was resuspended in PBS to double the volume giving a 50 % solution of cells, which was transferred to 2 mL storage tubes (labelled 'R').

The supernatant from the Buffy Coat tube was aliquoted into conical 1.5mL tubes and spun at 13,000 rpm for 5 minutes to pellet the platelets. After this the supernatants were discarded and the pellets resuspended in 1 mL of PBS into one of the tubes. This tube was spun again at 16,060g for 5 minutes and the supernatant discarded. The final pellet was resuspended in 1 mL of PBS and transferred to a 2 mL storage tube, labelled as Platelets (PT). The Buffy Coat pellet was resuspended in 14 mL PBS and inverted to mix. Then spun at 180g / 920 rpm for 10 minutes. The supernatant was discarded into NaOH and 13 mL of distilled water added. The tube was then inverted for 3 minutes to shock-lyse the RBC, and then 1 mL of 10 times

PBS was added. The tube was then spun at 180g / 920 rpm for 10 minutes. The supernatant was discarded and the tube refilled to the 14 mL mark with PBS and spun at 100g / 686 rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 1 mL of PBS labelled as Buffy Coat (BC). The resulting tubes were then stored at -80°C.

Figure 10

Flow diagram to illustrate blood component separation protocol



2.8 Preparation of brain / tissue homogenates

2.8.1 *Homogenisation Buffer*

10 mM Tris, 150 mM NaCl, 1 % (Octylphenoxy) polyethoxyethanol (IGEPAL), 0.5 % deoxycholic acid, 0.1 % SDS, 5mM EDTA, pH 8 plus aprotinin and leupeptin at a final concentration of 4 μG/mL.

2.8.2 Sample preparation

Preparation of human brain and tissue homogenates was conducted in a class 1 bio-safety hood in a category 3 laboratory.

The required weight of tissue (usually 60-100 mG) was sampled and transferred to a pre-weighed 2 mL Eppendorf safe-lock tube, using a disposable scalpel and forceps, samples were kept chilled in a pre-chilled freezer block. All contaminated materials were disposed of into 2M NaOH.

Tissue samples were then homogenised in 4°C homogenisation buffer to a 10 % w/v homogenate using an Eppendorf micro-pestle. Insoluble crude tissue fragments were removed by spinning at 2000 rpm for 5 minutes at 4°C in an Eppendorf 5417R centrifuge. The supernatant was removed and stored at -20°C for further analysis.

2.8.3 Proteinase K treatment of blood / tissue homogenates

Proteinase K stock 20 mG/mL (Merck, Drayton UK) was diluted to 2 mG/mL and stored at 4°C. PK was added to blood or tissue samples to the final concentration required and samples were incubated at 37°C for 1hour in a heat block. Proteolysis

was halted by the addition of either Pefabloc SC (Roche, Basel, Switzerland) to a final concentration of 1 mM, or by the addition of PMSF (Sigma Aldrich, UK) to 1 mM.

2.8.4 Blood cell lysis buffer (BCL) recipe

0.32 M Sucrose, 10 mM Tris-HCL, 5 mM $MgCl_2$, 1 % Triton X100, pH 7.5. All reagents obtained from Sigma-Aldrich, UK.

2.9 Western Blotting

2.9.1 SDS-PAGE

Protein separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using NuPAGE Novex 10 % Bis-Tris gels (catalogue number NUP0302), Novex gel tank apparatus, MOPS SDS NuPAGE running buffer (catalogue number NUP0001-02) and sample buffers (catalogue number NP0007) all from Invitrogen Ltd, Paisley, UK.

Samples for analysis were transferred to screw-top tubes and an equal volume of 2 times sample buffer added. The samples were then heated to 100°C for 10 minutes in a heat block. The pre-cast gel was rinsed with distilled H_2O, the white strip and comb removed and the wells washed twice with running buffer. The gel was then loaded in the tank and the tank was filled with running buffer. The samples and molecular weight markers (Catalogue number LC5600, LC5602 Invitrogen Ltd, Paisley, UK) were loaded and run at 200 Volts constant for 50 minutes.

2.9.2 Western Transfer and ECL detection

SDS-PAGE separated proteins were transferred to polyvinylidene fluoride (PVDF) using NuPAGE transfer apparatus and buffers (catalogue number NUP0006-1, Invitrogen Ltd, UK). 100 mL of MeOH was diluted 1/5 in NuPAGE transfer buffer (Invitrogen Ltd, UK) and 2 pieces of 3 mm blotting paper per gel and transfer pads were equilibrated in transfer buffer for 20 minutes. One piece of PVDF membrane (Hybond-P, Amersham Pharmacia Biotechnology, Chalfont St.Giles, UK) was wetted in MeOH and washed for 10 minutes in distilled H_2O prior to equilibration in transfer buffer. The SDS gel cassette was opened, and a piece of blotting paper placed on the gel. The PVDF membrane was placed on the other side and the gel placed into the transfer apparatus between several transfer pads. The transfer block was then filled with transfer buffer and the outer tank with distilled H_2O to act as a coolant. The transfer was then processed at 30V constant for 1hour.

The membrane was then washed twice for 3 minutes in 40 mL Tris-buffered saline with 0.1 % Tween 20 (Sigma- Aldrich, UK) (TBST) and blocked for 45 minutes in 50 mL 5 % milk powder in TBST. The membrane was washed 3 times as before in TBST followed by incubation with MAb 3F4 (Dako, Cambridgeshire, UK) at 1:1000 in 40 mL TBST for 45 minutes. The membrane was then washed a further 3 times prior to incubation with HRP anti-mouse IgG (Diagnostics Scotland, Edinburgh, UK) at 1:1000 in 40 mL TBST. The membrane was washed a further 4 times in TBST and the fluorescent signal developed by incubating the membrane with ECL PLUS (Amersham Pharmacia Biotechnology, Chalfont St.Giles, UK) for 5 minutes. Fluorescence imaging was visualised using the Storm 860 phosphoimager

(Molecular Dynamics, Amersham Pharmacia Biotechnology, UK) or exposed (e.g. 30 seconds, 3 minutes, 30 minutes) to Hyperfilm ECL (Amersham Pharmacia Biotechnology, RPN3103K) and developed using a Hyperprocessor (Amersham Pharmacia Biotechnology, UK).

2.10 Antibodies

A variety of different anti-prion protein monoclonal antibodies (MAb) have been employed in DELFIA, CDI, and western blotting studies described here. The table below summarises the name, reactivity, epitope specificity, and immunogen.

Table 6

Antibody	Epitope	Reactivity	Immunogen
FH11	46-58; 90-101	Bovine, Ovine, Human	Rec Ovine PrP
D18	133-157	Murine, Hamster, Human	Hamster PrP27-30
1120-64-9	175-230	Human	Purified human PrP^c
3F4	109-112	Bovine, Ovine, Human	263k Hamster
PV-30	96-105	Bovine, Ovine, Human, Murine	Bovine PrP

2.11 General Health and Safety

Procedures were carried out in accordance with guidelines for work with the agents of transmissible spongiform encephalopathies (TSEs) published by the Department of Health Advisory Committee on Dangerous Pathogens (ACDP) Spongiform Encephalopathy Advisory Committee (SEAC) joint working group (www.doh.gov.uk/cjd/tseguidance).

All procedures using infectious tissues and fluids were conducted in Class 1 bio-safety hoods in a Category 3 laboratory wearing gown/apron, protective footwear, protective eyewear and double gloves. Aerosol-resistant pipette tips and safe-lock eppendorf tubes were used. Centrifuge rotors were loaded and unloaded in a class 1 hood, not in the centrifuge. All contaminated plastic wear was immersed in 2N NaOH overnight. All contaminated liquids were brought to 2N NaOH and left overnight. Sawdust was added to NaOH decontaminated liquids, the container sealed and placed in yellow bags for incineration. Any accidental spills were treated by absorption on paper towel and NaOH decontamination.

2.12 Statistical Analysis

All statistical analysis including t-tests was carried out using NCSS 2001 program and Microsoft Excel. Box plots were produced by the NCSS 2001 software according to a common procedure in which boxes represent the interquartile range (IQR), the top and bottom of the box are the 25^{th} and 75^{th} percentiles, and the horizontal line through the box represents the median. The line and bar (whiskers) above and below the box represent the upper and lower adjacent values. The upper adjacent value is the largest observation that is </= to the 75^{th} percentile plus 1.5 times the IQR. The lower adjacent value is the smallest observation that is >/= to the 25^{th} percentile minus 1.5 times the IQR. Outliers, shown as small circles, are those values which lie outside the upper and lower adjacent values [153].

Chapter 3: Study of variation of prion protein in the peripheral blood of patients with variant and sporadic Creutzfeldt-Jakob disease detected by dissociation enhanced lanthanide fluoroimmunoassay and flow cytometry.

3.1 Plan

The first objective was to set up a DELFIA assay suitable for the screening of whole blood and separated blood components from clinical patients and healthy adult blood donor and neurological controls for the detection of PrP^c.

3.1.1 DELFIA assay calibration and standardisation

3.1.1.1 Comparison and calibration of platelet standard reagents

The DELFIA assay design for screening of whole blood and separated blood components was based upon methods developed previously by MacGregor et al [9]. In order to facilitate its successful transfer to the National CJD Surveillance Unit recalibration and standardisation of coating and detection antibodies and assay set-up was essential to ensure assay reproducibility. In addition an assessment of suitable conditions for the screening of whole blood and separated blood components was needed to ensure accurate quantitation of PrP^c.

As a source of PrP^c as a standard for assays, a lyophilised platelet standard reagent supplied by Perkin Elmer Life sciences was titrated against lyophilised platelet standards (NSL standards) used routinely by MacGregor et al [9] for assay calibration. Five assays were performed using plates precoated with monoclonal antibody FH11 supplied by Perkin Elmer Life sciences, UK (P820205), and using europium labelled

3F4 detection antibody at a 1/500 dilution (Lot 070201) also supplied by Perkin Elmer Life sciences. Assays were performed following the standard operating procedure described in the methods section 2.3 to provide an accurate evaluation of the concentration of PrP^c in the Perkin Elmer standard reagent. Both standards were diluted in DELFIA assay buffer at five fold dilutions: 1:5, 1:25, 1:125, 1:625, and 1:3125 and were assayed in duplicate. The NSL standard reagent had been previously titrated against human recombinant PrP with a ratio of 1 Unit (U) to 200 pG [150] and was assigned an equivalent concentration of 2188 U/mL. Dilutions of this standard were used to provide a standard curve against which the Perkin Elmer standard was calibrated. The plate layout for the assays is illustrated in Table 7; samples were assayed at several dilutions to ensure parallelism and accurate determination of concentration of PrP^c. The results are illustrated in Table 8.

Table 7

	1	2	3	4	5	6	7	8	9	10	11	12
A			3125	3125	625	625	125	125	25	25	5	5
B			3125	3125	625	625	125	125	25	25	5	5
C			3125	3125	625	625	125	125	25	25	5	5
D			3125	3125	625	625	125	125	25	25	5	5
E												
F												
G												
H												

Dilution	Concentration U/mL
5	437.5
25	87.5
125	17.5
625	3.5
3125	0.7

Plate map of 96 well microtitre plate showing layout of dilutions of NSL standard (rows A,C) and Perkin Elmer standard (B,D) for titre experiments. The smaller table below provides values for the calibration curve.

WorkOut software version 1.5 provided by PerkinElmer Lifescience was programmed to calibrate each assay. The means of sample duplicates were calculated, and blank means were subtracted before calculation of PrP^c concentration. The coefficient of variation (CV) was calculated to ensure good reproducibility between duplicate wells.

Table 8

Group	Wells	Raw data Mean	Blank Corrected	Concentration	% CV
Standard 1	A3, A4	797	380	0.7	2.48
Standard 2	A5, A6	1899	1482	3.5	0.15
Standard 3	A7, A8	8137.5	7720.5	17.5	1.59
Standard 4	A9, A10	45441	45024	87.5	2.62
Standard 5	A11, A12	243400	242990	437.5	2.87
Blank	A1, A2	417	0	0	0
PE Blank	B1, B2	436	10	0.03	4.22
PE 3125	B3, B4	1175.5	758.5	1.64	38.79
PE 625	B5, B6	2471	2054	4.94	4.69
PE 125	B7, B8	10817	10400	22.94	1.02
PE 25	B9, B10	58643	58226	110.78	2.85
PE 5	B11, B12	326390	325973	579.59	2.56
NSL Blank	C1, C2	443	26	0.06	0
NSL 3125	C3, C4	939.5	522.5	1.03	17.84
NSL 625	C5, C6	3496.5	3079.5	7.4	9.32
NSL 125	C7, C8	7443.5	7026.5	16.06	1.72
NSL 25	C9, C10	41565	41148	80.01	1.55
NSL 5	C11, C12	206140	205723	373.24	0.77
PE Blank	D1, D2	684	264	0.46	0
PE 3125	D3, D4	900	483	0.93	4.56
PE 625	D5, D6	2469.5	2052.5	4.94	1.69
PE 125	D7, D8	11610	11193	24.51	8.92
PE 25	D9, D10	59507	59090	112.34	1.7
PE 5	D11, D12	322630	322213	573.25	1.57
Blank	E1-H12				

Data for calibration assay 1, values in cpm are shown for raw duplicate mean and blank corrected values for each sample assayed. The calibrated concentration is expressed in U/mL and the coefficient of variation (CV) as a percentage. PE : standard supplied by Perkin Elmer, NSL: standard supplied by National Science Laboratory.

Table 9

Run1	A	B
PE 3125	1.64	5125
PE 625	4.94	3087.5
PE 125	22.94	2867.5
PE 25	110.78	2769.5
PE 5	579.59	2897.95
	Mean	3349.49
Run2		
PE 3125	0.93	2906.25
PE 625	4.94	3087.5
PE 125	24.51	3063.75
PE 25	112.34	2808.5
PE 5	573.25	2866.25
	Mean	2946.45

Table showing the concentrations of PrP^c as calculated by the calibrated DELFIA assay (A), and these values corrected by the dilution factor (B) and the overall mean for each of two runs of standard dilutions.

The concentration of each standard was calculated by correcting the calibrated value by the dilution factor, then calculating the mean of the dilution series as shown in table 9. Calibration of the Perkin Elmer standard reagent produced values of 3349.49 U/mL, and 2946.45 U/mL for each respective run. This gave an average calibration value of 3147.97 U/mL. Recalibration of the NSL standard gave a value of 2743.54 U/mL. The concentration of PrP^c present in the Perkin Elmer standard is therefore not dissimilar to the concentration of the NSL standard. Some variation in concentration exists between dilutions, but this is to be expected when dilution factors are high and concentration values are relatively low.

The Perkin Elmer standard was re-titrated against the NSL standard reagent a further three times giving the following values: 2570 U/mL; 2943 U/mL; 2090U/mL. The mean value generated from these 5 separate experiments was 2800U/mL, a value used as this standard reagent was used for assay calibration. New standards were

calibrated against this before use but calibration for assays and data described here were performed using the PerkinElmer standard reagent.

3.1.1.2 Calibration of platelet standard reagent against human recombinant PrP^c

In addition to the introduction of new standard reagents, the standard was calibrated against human recombinant PrP^c. Prionics recombinant PrP^c was assayed in duplicate in a dilution series from 5000-0.78 nG/mL, the assay was calibrated using a lyophilised platelet standard supplied by Diagnostics Scotland, UK which had been previously calibrated against NSL and Perkin Elmer standard reagents and assigned a value of 2530 U/mL. Concentrations of dilutions of recombinant PrP^c within the range of the calibration curve were converted to nG/mL, these values did not reflect the concentrations of recombinant PrP^c known to be present in each dilution (Table 10). The calibration was adjusted using the equation below to ensure that the concentration of PrP^c calculated reflects the concentration recombinant PrP^c assayed. Based on these findings 1 Unit = 25.76 pG/mL.

Table 10

Prionics Recombinant PrP^c Concentration nG/mL	Assay concentration U/mL	Concentration nG/mL
12.5	485.18	97.04
6.25	242.68	48.54
3.125	101.55	20.31
1.56	46.76	9.35
0.78	23.35	4.67

Determination of the concentration of PrP^c in nG/mL present in 1 U, calibrated using Prionics recombinant PrP^c

(485.18 * X) / 1000 = 12.5 nG;

X = (12.5 * 1000) / 485.18

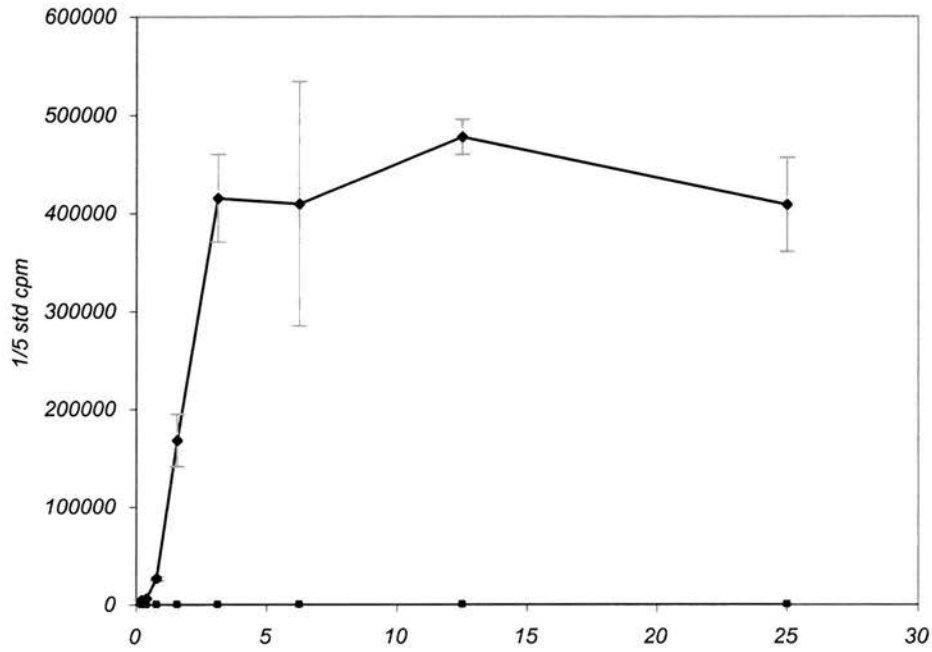
X = 25.76 pG

3.1.1.3 Titration of coating antibody

Coating plates with FH11 as an alternative to purchasing precoated microtitre plates from PerkinElmer Life sciences was explored in an attempt to reduce expenditure. MacGregor et al [9] previously recommended coating of 96 well microtitre plate with 5 μg/mL of monoclonal antibody. To ensure maximal binding and the sensitive detection of PrP^c rows of a microtitre plate were coated with a range of dilutions of FH11 in doubling dilutions between 0.195 – 25 μg/mL. Following overnight coating the plate was washed and incubated with a 1/5 dilution of platelet standard in DELFIA assay buffer, except the first two columns which were incubated with assay buffer alone to assess background binding. Five duplicates of a 1/5 dilution of platelet standard assessed the level of binding for each concentration of coating antibody. The assay was performed following the standard DELFIA protocol for detection of PrP^c using europium labelled 3F4 batch 29/10/2003 at a 1/15,000 dilution. The mean europium counts per minute for samples assayed at each concentration of coating antibody along with the two times standard deviation, to equal 95% confidence interval, were calculated and the data plotted (Figure 11). The concentration of FH11 at which the curve flattens off is the point at which further increases of coating antibody cease to increase the binding and the point at which coating has reached saturation. The previous assessment of 5 μG/mL by MacGregor et al [9] proved to be correct by this titration, the curve flattened off at 3.125 μG/mL,

and a coating concentration of 5 μG/mL slightly in excess of this would ensure maximal binding affinity.

Figure 11



Assessment of coating conditions for monoclonal antibody FH11. Concentration of FH11 (μG/mL) on the x-axis and detection of 1/5 dilution of platelet standard in counts per minute shown on the y-axis, the error bars show mean ± 2 sd of the 5 duplicate measurements.

3.1.1.4 Comparison of Dynatech Immunlon-4 HBX and Greiner microlon microtitre plates

In a continuation of experiments to standardise conditions for PrP^c analysis by DELFIA a comparison was made between two different plate types to assess whether medium or high binding plates were best suited for this type of analysis. Dynatech Immulon-4 high binding flat bottomed plates (those used routinely by

MacGregor et al [9]) and Greiner Bio-One Microlon medium binding plates (product No. 655080 Greiner Bio-ONE, UK) were coated overnight with 5 μG/mL FH11 and the standard DELFIA protocol for the detection of PrP^c described in section 2.3 was followed. Microtitre plates were incubated with dilutions of platelet standard, a plasma quality control sample assayed at 1/5, 1/10, and 1/50 dilutions, blank wells for an assessment of background binding, and 1/10, 1/20, and 1/40 dilutions of either untreated whole blood or the same whole blood treated with two episodes of snap freeze-thawing in dry ice and ethanol. The mean europium counts per minute and standard deviations for blank wells were calculated to give an indication of background binding. The concentration of the platelet standard was calculated to assess accuracy of measurement. The flagging of sample duplicates with a CV greater than 10 % was monitored to give an indication of binding reproducibility in duplicate wells.

Table 11

Dynatech Immulon-4 plates		sd
Blank Mean (n=10) cpm	557	+/- 28.94
Mean concentration Platelet standard U/mL	2616	+/- 41.10
Number flagged Duplicates	4	
Mean concentration Untreated whole blood U/mL	429	+/- 90.98
Mean concentration Freeze-thawed whole blood U/mL	590	+/- 67.54
Greiner Microlon plates		
Blank Mean (n=6) cpm	2228	+/- 248.86
Mean concentration Platelet standard U/mL	2309	+/- 186.75
Number flagged Duplicates	12	
Mean concentration Untreated whole blood U/mL	298	+/- 71.80
Mean concentration Freeze-thawed whole blood U/mL	443	+/- 89.96

Assessment of the suitability of two different microtitre plates for DELFIA, Blank means are shown in cpm and concentrations for platelet standard reagent and untreated and freeze-thawed whole blood expressed as a concentration in U/mL, standard deviations are also shown for each calculation.

The results summarised in table 11 show that the Dynatech Immulon-4 HBX plates are more suitable for DELFIA screening purposes. The background binding to these plates is a quarter that obtained using the Greiner plates, and is similar to background levels obtained using the FH11 plates precoated by PerkinElmer. The standard deviation measurement indicates that the blank wells show less variability than is seen with the Greiner plates. Calibration of the platelet standard was closer at 2616U/mL in Immulon-4 plates to the previously calibrated value of 2800U/mL in Perkin Elmer pre-coated plates than that of 2309U/mL obtained using the Greiner plates. The numbers of duplicates flagged due to a CV in excess of 10 % were three

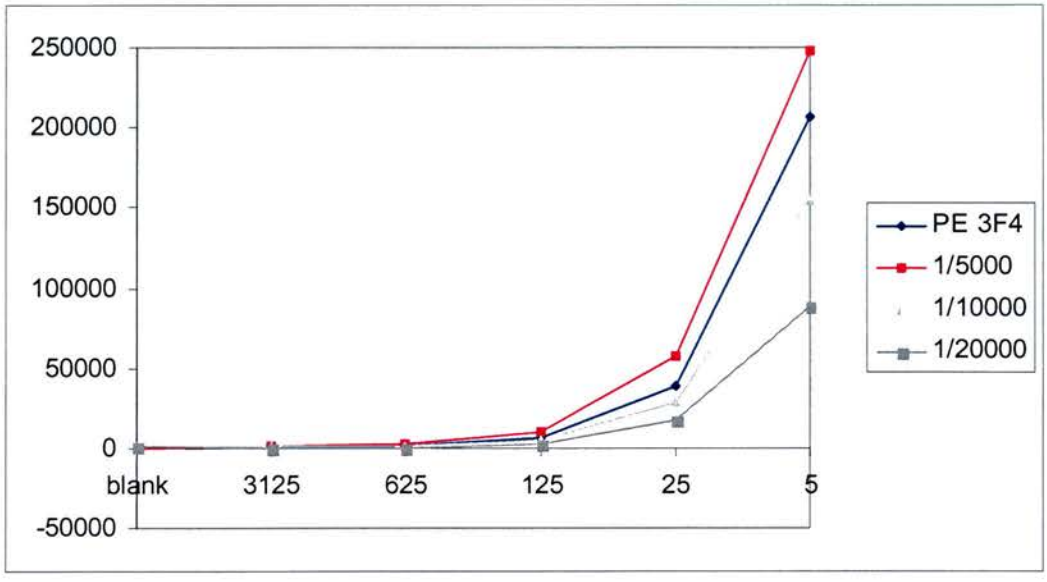
times higher using the Greiner plates than using the Dynatech plates. Finally higher levels of PrP^c detected in untreated and freeze-thawed whole blood samples were obtained using the Dynatech plates than with the Greiner plates. It was decided therefore to use the Dynatech microtitre plates for future DELFIA assays.

3.1.1.5 Titration and evaluation of europium labelled 3F4

DELFIA assays used europium labelled 3F4 monoclonal antibody supplied by PerkinElmer life sciences as detection antibody. This was supplied and labelled by PerkinElmer life sciences with a yield of 12 molecules of europium per IgG molecule, and recommended for use at a 1/500 dilution with filtration through a 0.22 μM filter. Given that this reagent was in short supply and expensive we explored the possibility of labelling and using our own antibody to reduce costs. 1 mG of 3F4 antibody obtained from the TSE Resource Centre Compton, UK was labelled (batch 11/7/02) following methods described in the materials and methods section 2.3.2 giving a yield of 24 molecules of europium per IgG molecule. The high labelling yield was probably a consequence of losses of IgG on the Microcon filter membranes given that the final protein content of labelled antibody was 0.47 mG/mL from 1 mG added at the beginning.

The labelled antibody was evaluated firstly by testing at 1/5000, 1/10,000, and 1/20,000 dilutions in parallel with current PerkinElmer labelled antibody for the detection of PrP^c in dilutions of the standard reagent. The detection in cpm of binding of each dilution of newly labelled 3F4 was plotted alongside the currently used PerkinElmer labelled 3F4 to determine which dilution performed similarly to the PerkinElmer supplied reagent. (Figure12).

Figure 12



Graph representing the detection in cpm (y axis) of PrP^c present in dilutions 1/5-1/3125 (x-axis) of platelet standard reagent using both the PerkinElmer labelled 3F4 (PE 3F4) and newly labelled 3F4 batch 11/7/02 at 3 different dilutions. All sample dilutions assayed in duplicate.

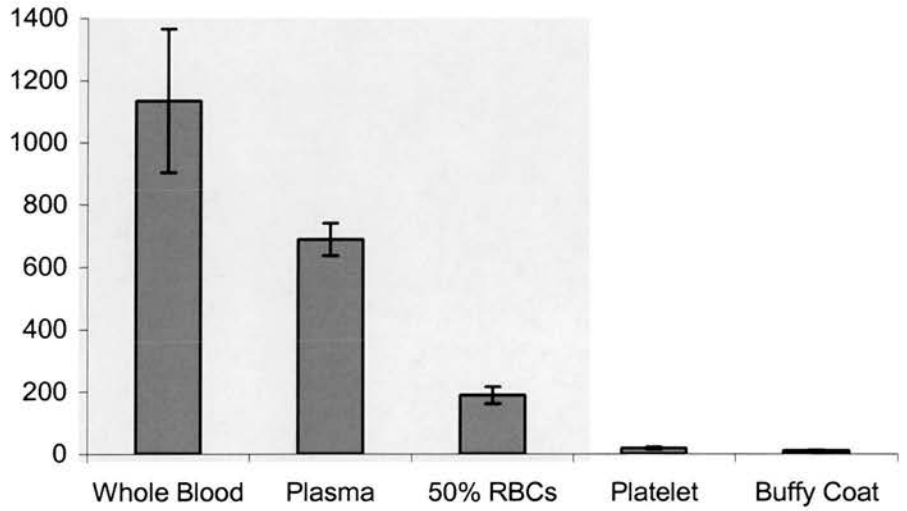
Given that the 1/5000 dilution of newly labelled 3F4 produced a calibration curve with higher europium counts at each dilution of standard reagent with similar background measurements to values obtained with the 3F4 labelled by Perkin Elmer this newly labelled 3F4 was used under these conditions. In general when newly labelled detection antibodies were characterised the conditions that produced counts >250000 for the 1/5 dilution of platelet standard coupled with a low background binding and good parallelism between dilutions were adopted as standard.

The second stage of evaluation of the labelled antibody was to assess its suitability for the detection of PrP^c in whole blood and blood components.

Whole blood and separated components including plasma, buffy coat, platelets, and 50 % Red Blood Cells from a healthy adult donor obtained from the Scottish National Blood Transfusion Service were assayed for PrP^c each at doubling dilutions

between 1/5-1/80. The assay was calibrated using lyophilised platelet standard obtained from Diagnostics Scotland and a plasma quality control sample was included for PrP^c assessment. The calibrated concentration in U/mL for each component was corrected for the dilution factor and the means and standard deviation were calculated. The CV for sample duplicates was also monitored to ensure it did not exceed 10 %. The chart below illustrates the mean concentrations and standard deviation for values of PrP^c present in each blood component.

Figure 13



Bar chart illustrating the mean PrP^c concentration in U/mL (y-axis) in whole blood and separated blood components from a healthy adult donor. The ± 2 sd for the calculation of PrP^c by 5 dilutions is shown as error bars.

The assay demonstrated good parallelism between dilutions with low standard deviations, and sample duplicates did not have CV that exceeded 10 %.
These conditions for use of labelled 3F4 were adopted for future assays for the detection of PrP^c.

3.2 Experiments to establish conditions for screening whole blood and separated components, effects of anti-coagulants, and the suitability of the blood separation protocol

Healthy adult controls in addition to all recieved clinical CJD samples to the CJD Surveillance Unit were separated using the blood separation protocol described in the material and methods section 2.7. The following experimental studies were conducted in order to determine the effects of freeze-thawing upon the detection of PrP^c, and the range of dilutions most suitable for each component to ensure parallelism between dilutions and the accurate quantitation of PrP^c. Experiments standardising conditions for DELFIA screening for PrP^c have included an analysis of untreated and freeze-thaw treated blood components, and data suggests that freeze-thaw treatment causes an increase in the levels of PrP^c detectable in peripheral blood. In addition to these studies full blood count and flow cytometry studies were carried out on several healthy adults to determine the effects of blood anticoagulants upon PrP^c detection and the purity of separated blood components.

3.2.1 Comparison of refrigerated and freeze-thawed separated blood components

A 10 mL citrate anti-coagulated whole blood sample from a healthy adult donor was separated and buffy coat, 50 % red blood cells, platelet, and plasma components were split into 2 aliquots one of which was stored frozen at -80° C and the other refrigerated at + 4° C for 24 hours. Each component aliquot was diluted to 1:5, 1:10, 1:20 in DELFIA assay buffer and assayed in duplicate for PrP^c by DELFIA. The

assayed concentrations were corrected by the dilution factor and the means and standard deviations were calculated for each component, the results are listed in table 12. The highest concentrations of PrP^c were detected in freeze-thawed aliquots compared with refrigerated aliquots. This result was largely expected since PrP^c is expressed on cell membranes as well as in the cell cytoplasm. The action of freeze-thaw lysis will permit the detection of internal cell PrP^c and will disrupt the association of PrP^c with cell membranes, therefore making more available for detection. These findings though may also be a consequence of PrP^c degradation in refrigerated samples. The levels of PrP^c detected in different blood fractions are largely as expected with highest levels detectable in the plasma and buffy coat components, with lower levels in the Red Blood Cell fraction. What was not expected was the low concentration of PrP^c detectable in the platelet component however this may be a consequence of the separation protocol. Where PrP^c levels are high for example in plasma and frozen buffy coat components there is good parallelism between dilutions, however where levels are low for example in Red Blood Cell components it would be advisable to assay these components undiluted or at lesser dilutions.

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Table 12

Blood Component	Concentration PrP^c	Concentration U/mL	Mean of 3 dilutions	Standard Deviation
Frozen Buffy Coat 1/5	8.23	41.15	-	-
Frozen Buffy Coat 1/10	3.21	32.10	-	-
Frozen Buffy Coat 1/20	1.25	25.00	32.75	8.09
Ref Buffy Coat 1/5	0.43	2.15	-	-
Ref Buffy Coat 1/10	0.77	7.70	-	-
Ref Buffy Coat 1/20	0.20	4.00	4.62	2.83
Frozen RBC 1/5	3.98	19.90	-	-
Frozen RBC 1/10	*4.00	40.00	-	-
Frozen RBC 1/20	0.46	9.20	14.55	7.57
Ref RBC 1/5	0.34	1.70		
Ref RBC 1/10	0.21	2.10		
Ref RBC 1/20	0.20	4.00	2.60	1.23
Frozen Plasma 1/5	56.80	284.00	-	-
Frozen Plasma 1/10	27.60	276.00	-	-
Frozen Plasma 1/20	13.70	274.00	278.00	5.29
Ref Plasma 1/5	49.53	247.65	-	-
Ref Plasma 1/10	22.14	221.40	-	-
Ref Plasma 1/20	10.85	217.00	228.68	16.57
Frozen Platelets 1/5	0.07	0.35	-	-
Frozen Platelets 1/10	0.03	0.30	-	-
Frozen Platelets 1/20	0.00	0.00	0.22	0.19
Ref Platelets 1/5	0.02	0.10	-	-
Ref Platelets 1/10	0.00	0.00	-	-
Ref Platelets 1/20	0.04	0.80	0.30	0.44

Table showing results of DELFIA assay for detection of PrP^c in frozen and refrigerated (Ref) aliquots of buffy coat, RBC, plasma, and platelet components assayed at 3 dilutions.
** Signifies samples where the duplicate coefficient of variation was >10, these Figures were excluded from mean calculations.*

3.2.2 Variability of PrP^c detection amongst healthy adult controls

In order to study the degree of variability in PrP^c expression in healthy adult controls citrate anti-coagulated whole blood samples were separated and assayed for PrP^c by DELFIA at three dilutions in duplicate. The results are listed in Table 13 below.

Table 13

Separated Component	Mean PrP^c U/mL	N	Range U/mL	Standard Deviation
Buffy Coat	68	8	16-156	46.5
Plasma	460	10	357-540	43.2
50% RBC	13	8	6-19	4.6
Platelet	147	8	4-163	67.8
Whole Blood	484	6	289-680	171.2

Blood components from healthy adults (n) assayed by DELFIA for PrP^c (U/mL). All samples assayed in duplicate at 1:5, 1:10, 1:20.

The results compare well with the findings of MacGregor et al [9] with the exception of levels of platelet PrP^c which were lower, this is a probable consequence of differences in the preparation of blood components. Fresh citrate phosphate dextrose anti-coagulated whole blood components were prepared by a buoyant-density centrifugation method over polymorphprep by MacGregor et al [9] in contrast to the separation method used and described here. These differences may explain why MacGregor et al found the highest levels of PrP^c in the platelet component. The separation method used here was not ideal for isolation of the platelet and buffy coat components; cells often clumped together and proved difficult to resuspend, this may explain the large ranges in the platelet and buffy coat data. This protocol was designed to produce these components without the need to use Ficoll. Ficoll, like dextran sulphate, is a polyanion, and it was thought that it may interfere with the processing and replication of the infectious agent as has been reported for dextran sulphate [154]. It was necessary to employ a separation protocol that avoided Ficoll because blood from clinical CJD patients was archived not only for this study but also for future infectivity studies, and the use of Ficoll may have reduced infectivity levels in these samples.

It is possible that the reduced PrP^c level detected in platelet fractions was due to the effects on platelets of using citrate as an anticoagulant or cellular contamination of non-platelet components with platelets.

In order to evaluate these possibilities 30 mL of whole blood from a healthy adult donor was split into three vacutainers containing citrate, EDTA, and heparin anti-coagulants. These samples were then separated following the standard separation protocol, and whole blood, plasma, RBC, and platelet components were assayed for PrP^c by DELFIA. Full blood counts were performed on the same samples to provide cell counts and give an indication of cellular contamination of separated components. Table 14 outlines the findings which show that the use of different anti-coagulants has a significant effect upon whole blood platelet counts. Counts were at least two times higher using EDTA than citrated blood. The platelet count using heparin was particularly low which was expected considering heparin is known to cause platelet aggregation. Others (G.R.Barclay personal communication) have shown that the leucocyte-platelet adherence is much less in EDTA than in citrate or heparin, where platelets may adhere to leucocytes in buffy coat components. The justification for using citrate as an anticoagulant for clinical and control samples was based upon claims by Wadsworth et al [155] that copper chelation activity of EDTA led to changes in the structure of PrP. This was found as different cleavage patterns which gave rise to fragments of different molecular mass in western blotting studies. More recent studies by Notari et al [156] show however that it is the elevation in pH caused by the addition of EDTA and not the chelation activity that causes changes to cleavage

patterns. In hindsight EDTA may have been the more appropriate anti-coagulant given the effects of citrate on cell counts.

Table 14

Whole Blood Anticoagulant	Platelet count 10^9/L	RBC count 10^{12}/L	White cell count 10^9/L
Citrate	104	3.98	3.83
Heparin	53	4.37	5.49
EDTA	219	4.28	4.34

Full blood counts of whole blood samples in 3 different anti-coagulants

The effect of different anti-coagulants is evident when whole blood is separated into constituent components and assayed for PrP^c by DELFIA. The concentrations of PrP^c detectable in whole blood, plasma, and platelet components for each anticoagulant were calculated by assaying each component at three dilutions in duplicate, any duplicate with a CV greater than 10% was excluded. Red Blood Cell and buffy coat components were included in this analysis, PrP^c levels were at the limit of detection of the assay and thus could not be determined with accuracy and were excluded from final results analysis (Table 15). The use of citrate as an anti-coagulant causes a decrease in the concentration of PrP^c detectable by DELFIA in all components assayed compared with heparin and EDTA. The use of EDTA resulted in the detection of the highest concentrations of PrP^c in plasma and platelet components.

Table 15

Component / anti-coagulant	PrP^c U/mL	Stdev	Platelet count 10^9/L	PrPc U/10^9/L
Whole blood				
Citrate	388	35.23	-	-
Heparin	714	132.23	-	-
EDTA	436	6.81	-	-
Plasma			-	-
Citrate	496	102.53	-	-
Heparin	555	30.51	-	-
EDTA	733	101.3	-	-
Platelets			-	-
Citrate	139	19.09	64	2.17
Heparin	1353	67.36	151	8.96
EDTA	1804	77.07	306	5.90

Concentrations of PrP^c detectable by DELFIA in whole blood, plasma, and platelet components using different anti-coagulants. The mean concentration (U/mL) and sd are shown as well as platelet counts for the platelet components.

It is clear therefore that citrate is having an effect upon platelets causing a reduction in platelet numbers in the platelet component when whole blood samples are separated. This is unusual since platelet aggregation is usually associated with the use of heparin as seen in table 14, which causes inaccurate platelet cell counts when full blood counts are performed. EDTA is used routinely by haematology laboratories to investigate platelet cell counts in peripheral blood, because its use is not associated with aggregation. It is possible that the calcium binding activity of citrate is in some way responsible for decreases in the detection of PrP^c but the exact mechanism is unknown. What is clear though is that the effects of the separation protocol coupled with the use of citrate causes major decreases both in platelet numbers detected by full blood count analysis and in the concentration of PrP^c detectable in platelet components due to this reduction in cell numbers.

Despite these findings the use of citrate was still favoured as the anti-coagulant of choice; this ensured that results from analysis of all samples were directly

comparable, however the detrimental effects of citrate on platelet counts is evident in future results.

Full blood counting of separated components allowed an assessment of the purity of separated blood components and the suitability of the blood separation protocol. Citrate anti-coagulated whole blood from two healthy adults was separated into constituent components on which full blood counts were performed. Cell counts on separated components found there to be no cellular contamination of the plasma component, and no cells other than platelets were detected in the separated platelet component. The Red Blood Cell component was also relatively pure despite a small amount of leucocyte contamination 0 – 1.57 X 10^9/L which is relatively small considering there were > 10 X 10^9/L leucocytes present in unseparated whole blood samples. The separation protocol therefore produced components with good purity and little cellular contamination. However these studies demonstrated the very low levels of platelets and leucocytes present in respective platelet and buffy coat components once separated. Platelet counts were 2 X 10^9/L and 27 X 10^9/L in the platelet components compared with 56 X 10^9/L and 64 X 10^9/L in untreated citrate anti-coagulated whole blood samples. Leucocyte cell numbers in separated buffy coat components were undetectable in one control and 0.81 X 10^9/L in the second compared with 10.51 X 10^9/L and 10.19 X 10^9/L in untreated citrate anti-coagulated whole blood samples. Problems associated with the resuspension of cell pellets and problems with cell clumping associated with the use of citrate are likely to explain these low cell counts, and these problems are evident in assessment of PrP^c levels in platelet and buffy coat components from clinical CJD and control groups.

As a result of the finding of low concentrations of PrP^c detectable in Red Blood Cell, platelet and buffy coat components they were assayed in future at five doubling dilutions between 1/5 – 1/80. Where levels were particularly low samples were rescreened as undiluted samples. PrP^c was detectable at higher concentrations in whole blood and plasma components and so they were assayed at doubling dilutions in the range 1/10 – 1/160 in order to compensate for quenching evident at lower dilutions and to ensure good parallelism between each dilution series. Data was expressed in Units per mG total protein for separated cellular components to normalise data by compensating for the effects of the separation protocol upon cellular particulate recovery in components and to ensure that range differences in cell number did not contribute to differences in PrP^c levels between groups. Although studies on full blood counts of clinical patient samples and controls did not show abnormalities, full blood count data could not be generated from frozen stored clinical and control samples used in this study, therefore normalisation by total protein was essential for all components with the exception of whole blood.

The preliminary experiments presented so far represent studies designed to standardise DELFIA assay procedure and the titration of reagents used therein. These studies ensure that the assay is sufficiently developed and sustainable to ensure accurate and comparable measurement of PrP^c in whole blood and separated blood components from both clinical CJD patient and control groups to allow the investigation of potential differences.

3.3 Study of variation in concentration of prion protein in the peripheral blood of patients with variant and sporadic Creutzfeldt-Jakob disease detected by dissociation enhanced lanthanide fluoroimmunoassay and flow cytometry

3.3.1 DELFIA analysis

DELFIA technology was employed to study potential variation in the expression of PrP^c in vCJD and sCJD patients, healthy adult controls and neurological controls. Whole blood separation and the DELFIA PrP^c assay were carried out following methods detailed above (2.3, 2.7). The means, median and standard deviation were calculated and the Mann-Whitney U test was used to determine the significance of differences in medians between groups. Data was expressed in Units per mL total protein, for whole blood and all separated components, to normalise data by compensating for the effects of the separation protocol upon cellular particulate recovery in components, and to ensure that range differences in cell number did not contribute to differences in PrP^c levels between groups. Although full blood count data from clinical patient samples and controls did not show gross abnormalities, full blood count data could not be generated from frozen stored clinical and control samples used in this study, therefore normalisation by total protein was essential for all components with the exception of whole blood.

Measurements of total protein levels were carried out using a Bio-Rad protein assay as described in section 2.6.

3.3.1.1 *Detection of whole blood PrP^c by DELFIA*

Whole blood samples from 10 patients with vCJD, 10 patients with sCJD, 8 neurological controls, and 29 healthy adults (1 excluded due to lack of correlation between dilutions) were analysed in duplicate at 5 dilutions by DELFIA. For each group the median, and interquartile range was calculated and data expressed in U/mL (Figure 15A, Table 16). There is a significant decrease in the concentration of PrP^c in vCJD ($p=0.005$) and neurological control patients ($p=0.0001$) compared with healthy adults, but not between vCJD and sCJD, nor between neurological controls and sCJD patients. Despite the significance there was considerable overlap between the vCJD and healthy adult control groups which indicates that this observation would have little use as a discriminatory test for diagnosis or screening. These differences between groups remain in the same relative order and significance when PrP^c concentration is normalized for total protein and expressed as U/mG; there remains a significant decrease in the concentration of PrP^c in vCJD ($p=0.012$) and neurological control patients ($p=0.0004$) when compared with healthy adults (Figure 14A).

3.3.1.2 *Detection of platelet poor plasma PrP^c by DELFIA*

Platelet poor plasma samples from 10 patients with vCJD, 10 patients with sCJD, 29 healthy adults (1 excluded due to lack of parallelism between dilutions), and 6 samples (2 samples unavailable) from neurological controls were analysed by DELFIA for PrP^c (Figure 14B, Table 16). We found significant elevation in the plasma PrP^c concentration in sCJD patients when compared with both healthy adult ($p=0.022$) and neurological control groups ($p=0.050$), but not when compared to levels found in vCJD patients. No significant differences were found in comparisons

between controls and vCJD groups. Statistically significant differences between groups were not evident when data was expressed in U/mL PrP^c (Figure 15B)

3.3.1.3 Detection of Red Blood Cell PrP^c by DELFIA

50% Red Blood Cell samples from 10 vCJD patients, 9 sCJD patients, 26 healthy adults, and 7 neurological controls were analysed by DELFIA to determine PrP^c concentration (Figure 14C, Table 16). The neurological control group showed a significant reduction in PrP^c concentration when compared with vCJD patients ($p=0.029$), sCJD patients ($p=0.024$), and healthy adults ($p=0.001$). vCJD, sCJD patients and healthy adults all exhibited similar median values for PrP^c concentration and therefore did not exhibit significant differences when compared with each other.

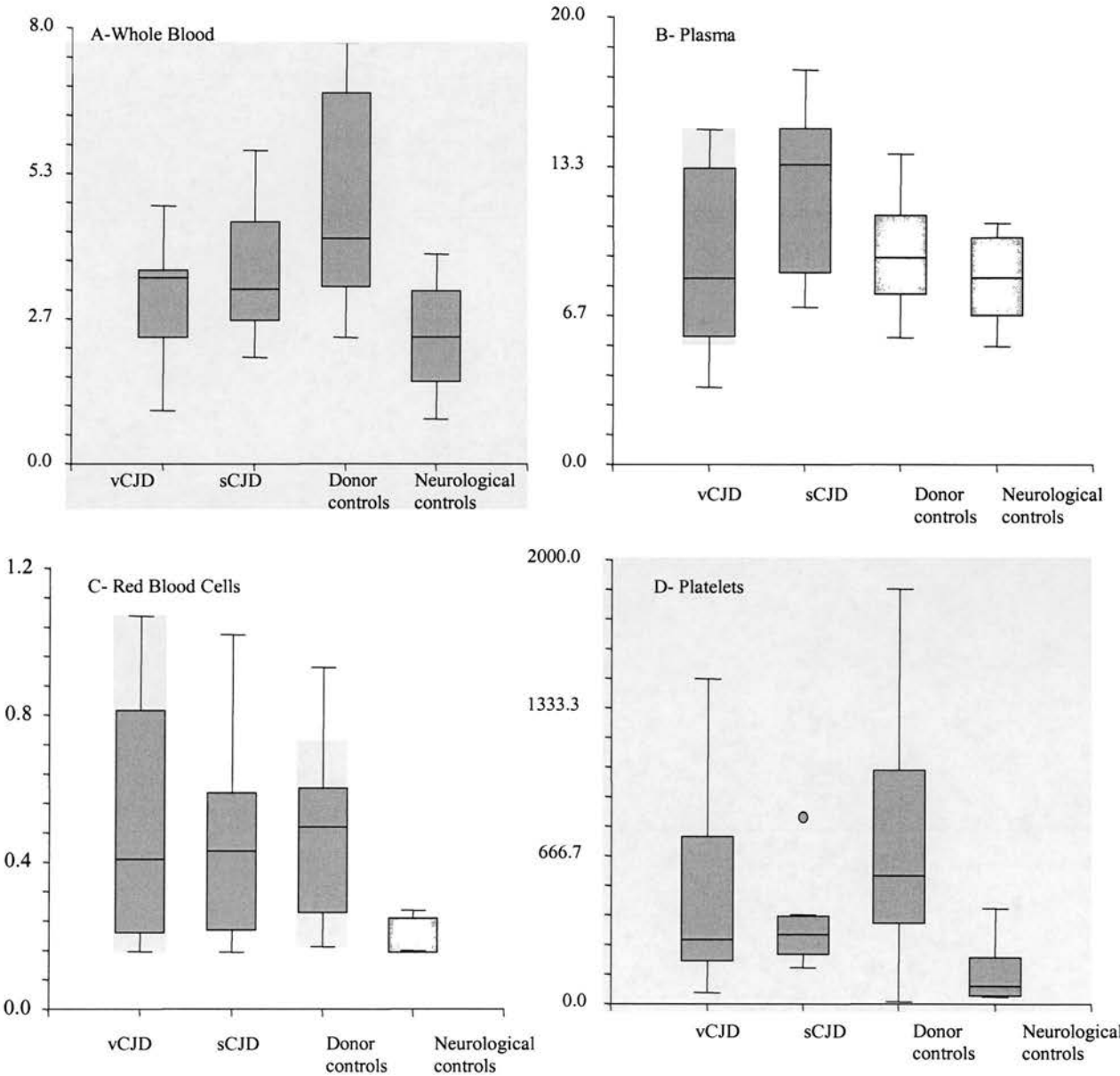
3.3.1.4 Detection of Platelet PrP^c by DELFIA

Platelet samples from 24 healthy adults, 9 patients with vCJD, 9 patients with sCJD, and 7 neurological controls were analysed for PrP^c concentration (Figure 14D, Table 16). Samples excluded from each group constitute those where PrP^c concentration could not be measured reproducibly across a range of dilutions. The level of platelet PrP^c in neurological controls was significantly reduced compared to levels in healthy adults ($p=0.001$), vCJD patients ($p=0.039$), and sCJD patient samples ($p=0.017$). In addition the concentration of platelet PrP^c in the sCJD samples was significantly reduced compared with levels in healthy adults ($p=0.021$) but not against vCJD.

3.3.1.5 *Detection of Buffy Coat PrP^c by DELFIA*

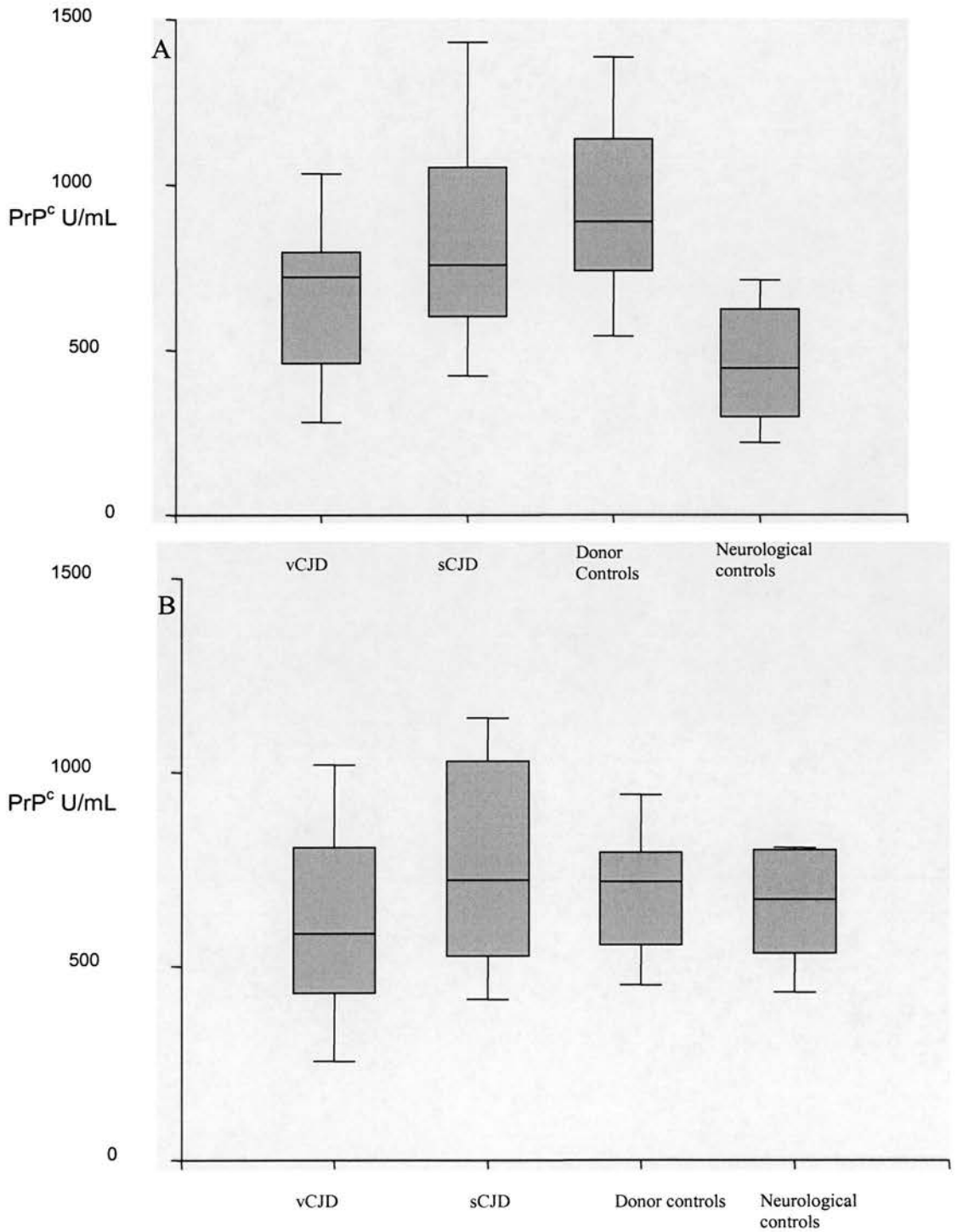
Buffy coat PrP^c expression levels were not detected at significantly high concentrations, particularly in clinical groups, to permit accurate measurement. Hence they were excluded from the analysis.

Figure 14



The concentration of PrP^c assayed by DELFIA in separated blood components of clinical and control samples. The concentration of whole blood (A), platelet poor plasma (B), 50% red blood cell (C), and platelet (D) PrP^c (Units per mG) assayed by DELFIA in vCJD, sCJD, and donor and neurological control groups.

Figure 15



The concentration of PrP^c assayed by DELFIA in separated blood components of clinical and control samples. The concentration of whole blood (A), platelet poor plasma (B), PrP^c (Units per mL) assayed by DELFIA in vCJD, sCJD, and donor and neurological control groups.

Table 16

Sample	Blood component	N	Median U/mG	Range 95% CL U/mG
vCJD	Whole Blood	10	3.438	1.912 - 3.593
sCJD	Whole Blood	10	3.227	2.073 - 5.754
Donor Controls	Whole Blood	29	4.16	3.376 - 6.635
Neurological Controls	Whole Blood	8	2.35	0.823 - 3.375
vCJD	Plasma	10	8.375	5.225 - 14.912
sCJD	Plasma	10	13.446	8.078 - 17.314
Donor Controls	Plasma	29	9.316	8.038 - 10.624
Neurological Controls	Plasma	6	8.405	5.31 - 10.8
vCJD	Red Blood Cells	10	0.411	0.19 - 1.045
sCJD	Red Blood Cells	9	0.434	0.162 - 0.648
Donor Controls	Red Blood Cells	26	0.499	0.29 - 0.579
Neurological Controls	Red Blood Cells	7	0.16	0.16 - 0.27
vCJD	Platelets	9	293.93	174.42 - 1150.442
sCJD	Platelets	9	316.56	213.69 - 400.75
Donor Controls	Platelets	24	581.11	389.29 - 855.15
Neurological Controls	Platelets	7	83.37	35.48 - 429.6

Table to illustrate the medians and range (95% confidence limits) in U/mL of separated components from different clinical and control groups as measured by DELFIA

3.3.2 Flow cytometry analysis

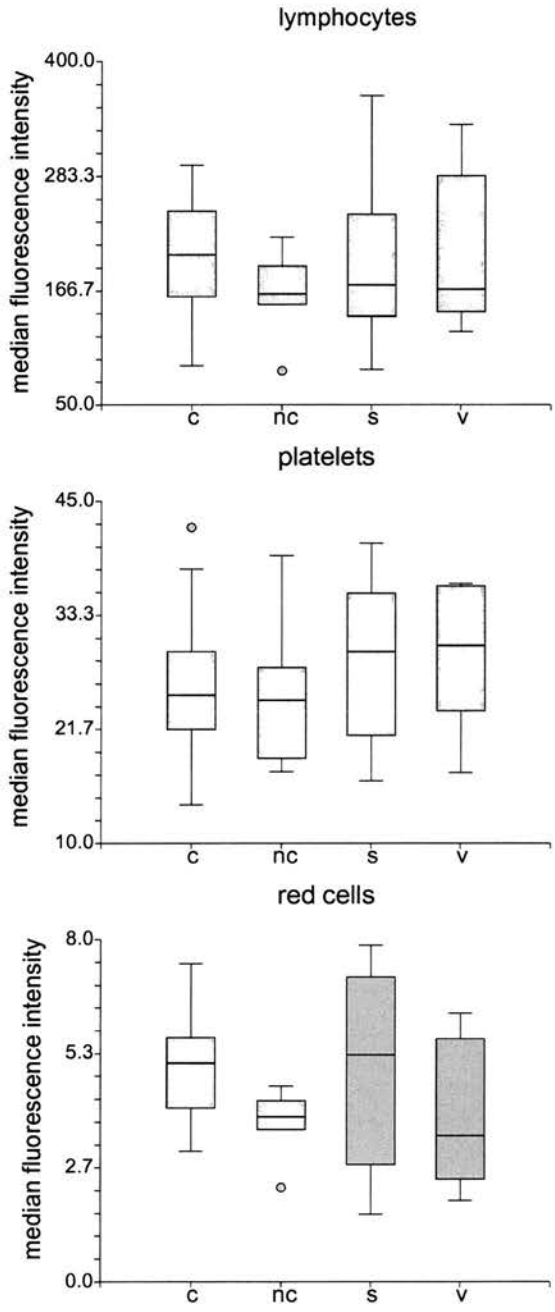
The cell associated PrP (net median fluorescence intensity above background) ranges for lymphocytes platelets and red cells are shown as interquartile box plots (Figure 16) for healthy adult controls, neurological controls, sCJD cases and vCJD cases. The only significant difference found between groups was between red cell PrP in healthy adults and in non-CJD neurological controls ($p = 0.008$). Although platelet PrP was raised in both sCJD and vCJD cases compared to healthy adults or neurological controls, this did not reach significance.

Clinical vCJD and sCJD cases were found to be as sensitive as healthy adults and non-CJD neurological patients to removal of cell-associated PrP by PK (Figure 17). The distribution of FITC fluorescence following PK treatment was homogeneous, showing a single low peak in the FITC channel without any discernible higher peak

which might indicate the retention of PK resistant PrP, putative PrP^{Sc}, on any subpopulation of cells.

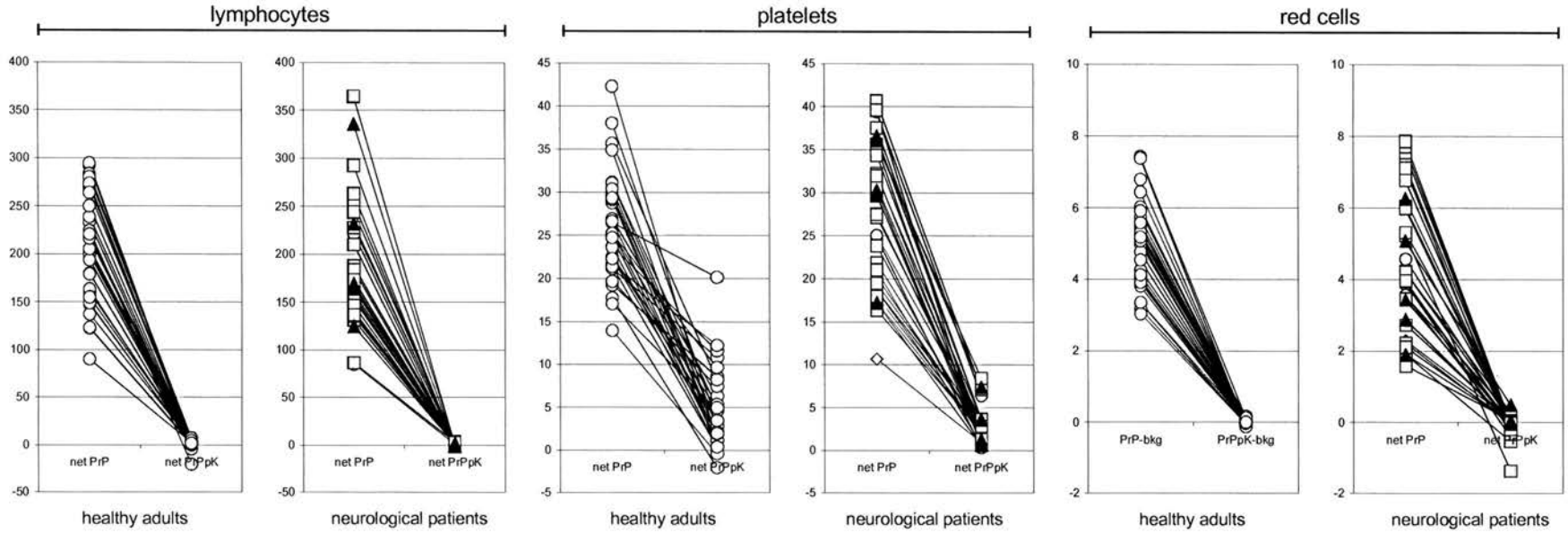
Results are not reported for neutrophils or monocytes since in these studies we considered the results unreliable. There appeared to be considerable non-specific binding of fluorescence by both leucocyte classes especially evident in both healthy adult controls and all clinical cases which may have been a consequence of sample age.

Figure 16



Box plots showing the ranges of expression of cellular PrP shown by flow cytometry on different clinical and control groups. Expressed as median fluorescence intensity net of background, on lymphocytes, red cells and platelets.

Figure 17

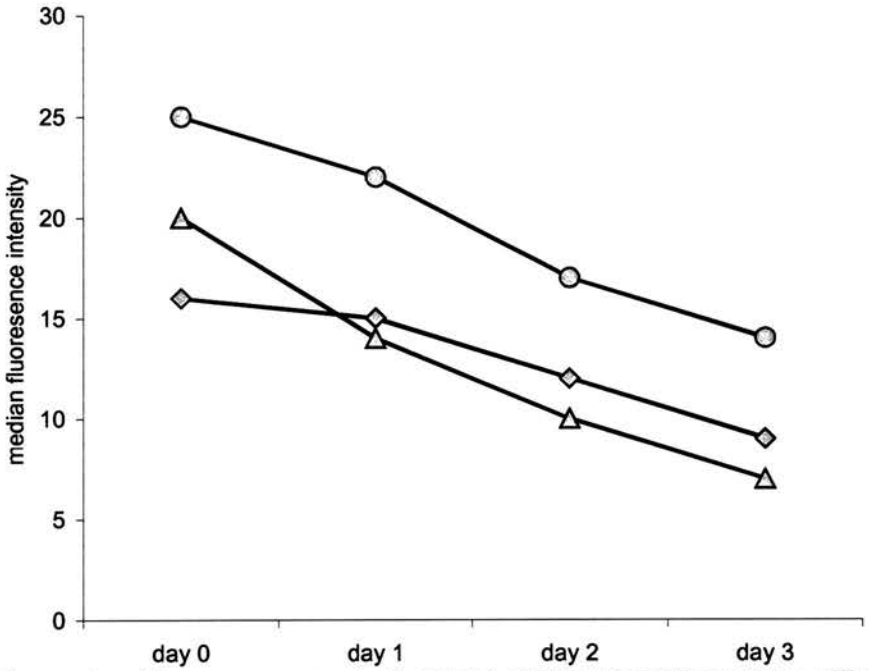


Demonstration by flow cytometry of the removal of cell surface PrP following PK treatment. The median fluorescence intensity (net of background) is plotted for cells pre- and post- PK treatment and connected by a line for each case. Key: open circles - healthy adult blood donors; shaded circles – non-CJD neurological patients; shaded squares - sCJD patients; black triangles – vCJD patient; open diamond – familial TSE (a single case of familial TSE was included in the study but not included in statistical analysis).

3.3.3 *Stability of whole blood PrP^c*

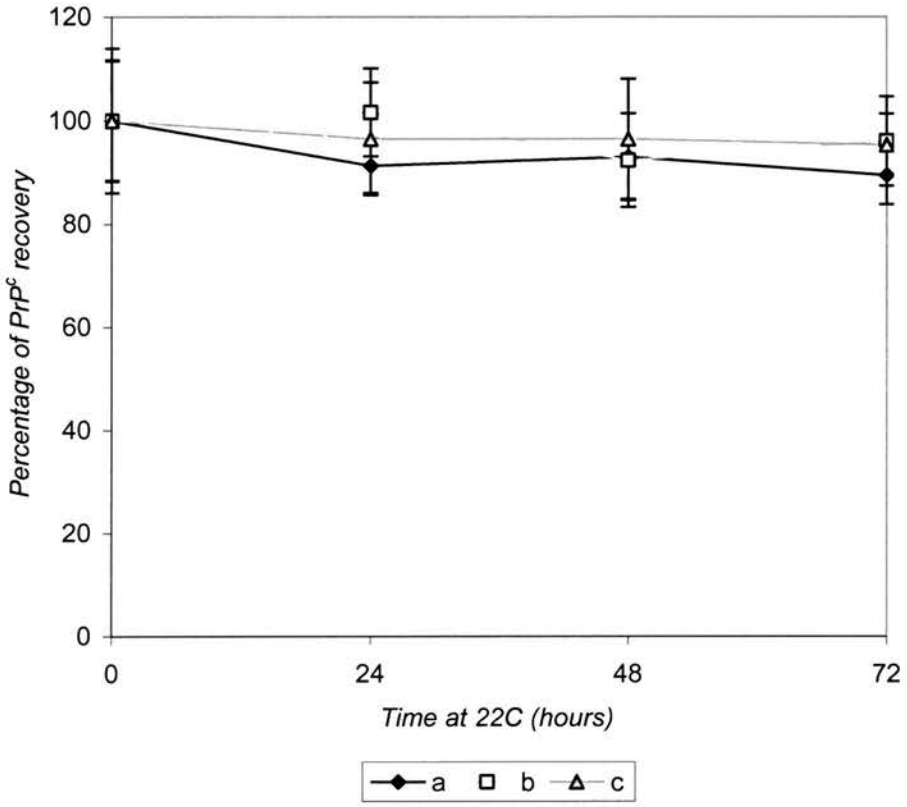
Some samples from clinical groups took longer than 24 hours to arrive in the laboratory. To consider any effects of prolonged transit time at ambient temperature, whole blood samples from 3 healthy adult controls were left at 22° C for 72 hours and samples were removed from each control at 24-hour intervals. Samples were analysed at each time point by flow cytometry, and 1mL samples for DELFIA analysis were stored at -80° C until analysis for the detection of whole blood PrP^c. The expression of PrP on platelets measured by flow cytometry at 24-hour intervals showed sequentially decreasing levels of PrP with time for each individual (Figure 18). This contrasted to identification of increased expression of PrP on platelets found in a preliminary analysis of data for sCJD patients compared with healthy adult controls, and implied sample storage was not responsible for this increase in the patient group. Levels of PrP^c detected by DELFIA remained stable across 72 hours (Figure 19). Prolonged transit time and ambient storage temperatures had negligible effects upon PrP^c concentration, and are therefore unlikely to contribute to differences between sample groups.

Figure 18



Flow cytometric measurement of platelet PrP in blood from three different healthy adults determined on a fresh sample and on the same sample on three successive days after sample storage at room temperature.

Figure 19

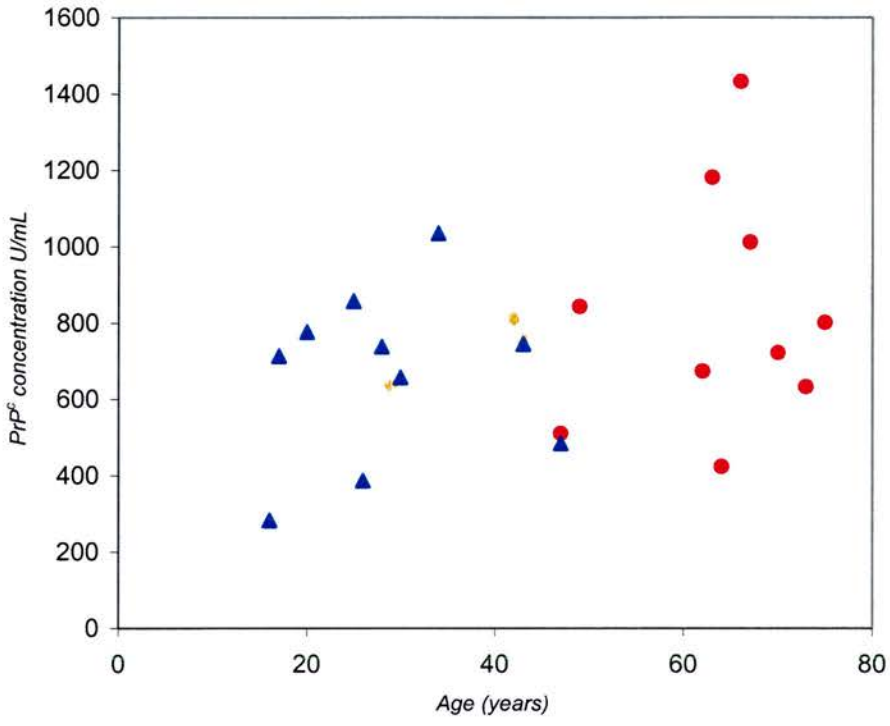


Stability of PrP^c in whole blood samples. Samples from 3 healthy blood donors (a, b, c) were used to investigate the effects of temperature and storage time on the detection of PrP^c. Samples were left at 22° Celsius for 72 hours. Samples were taken every 24 hours and stored at -80° Celsius until DELFIA analysis. The data points show mean percentage changes in PrP^c concentration, the error bars show ± 2 sd. Samples were assayed 3 times in duplicate.

3.3.4 Relationship between age and PrP^c levels

Differences between control and clinical sample groups may be affected by the age of individuals, considering vCJD usually affects young people and sCJD older people. The concentration of PrP^c (U/mG) in whole blood samples detected by DELFIA in CJD and control groups was plotted against age and showed that PrP^c expression is unrelated to this parameter (Figure 20). Linear regression analysis was used to show the lack of correlation shown as R- squared values in the legend.

Figure 20



Scatter graph to illustrate the lack of correlation between PrP^c concentration (Units per mL) and age (years) in vCJD (▲) R^2: 0.02, sCJD (●) R^2: 0.06, and apheresis donor control sample groups ( ) R2: 0.05.

3.3.5 Haematology of clinical and control samples

To ensure that haematological abnormalities in blood samples from clinical patients and controls did not contribute to differences in PrP concentration and expression between groups, full blood count data for clinical and neurological patients was reviewed. No gross abnormalities were detected. It was not possible to obtain haematology on the exact same sample taken for genetic analysis and subsequently separated and analysed for PrP^c by DELFIA; this data was obtained from patient hospital records closest to the time possible and is shown in Table 17.

Table 17

sCJD	Date 1	Date 2	Hb g/dL	WBC x10^9/L	Pltsx10^9/L	RBCx10^{12}/L	MCV fL
1452	19/09/2001	03/12/2001	15	6.2	223	5.15	87.4
1467	14/01/2002	21/01/2002	14.9	10.8	311	-	-
1472	07/01/2002	17/01/2002	15.4	8.3	243	4.95	92.2
1480	03/01/2002	04/02/2002	14.5	7.4	257	4.74	91
1489	10/01/2002	07/03/2002	13.4	4.5	228	4.77	85
1499	11/03/2002	15/03/2002	13.5	10.3	308	-	-
1508	04/03/2002	08/04/2002	15.5	12.7*	237	-	-
1517	08/04/2002	02/05/2002	11.9	7.7	190	-	-
1514	30/04/2002	13/05/2002	14.4	9.4	219		86
1529	01/05/2002	-	15.2	8.4	256	4.92	91.7
vCJD							
1421	18/09/2001	09/10/2001	16.6	5.6	239	5.4	-
1424	26/09/2001	15/10/2001	14.9	5	329	5.02	-
1416	-	25/10/2001	-	-	-	-	-
1458	-	29/11/2001	-	-	-	-	-
1475	01/01/2002	24/01/2002	14.4	6	264	4.86	-
1476	17/01/2002	05/02/2002	15.8	5.94	364	5.05	-
1509	20/03/2002	26/04/2002	14.3	8.5	260	4.56	-
1518	18/02/2002	07/05/2002	15.2	9.5	208	-	-
1526	10/05/2002	29/05/2002	15.7	10.22	319	5.4	83.4
1532	01/06/2002	17/06/2002	15.1	11.6*	404	-	-
1630	-	24/01/2003	-	-	-	-	-
non-CJD							
	06/06/2002	03/07/2002	13.1	7	422*	4.41	86
	25/07/2002	27/08/2002	10.6*	12.59*	449*	4.14	80
	-	28/11/2002	-	-	-	-	-
	-	09/04/2003	-	-	-	-	-
	-	04/07/2003	-	-	-	-	-
	-	13/11/2001	-	-	-	-	-
	-	12/12/2002	-	-	-	-	-
	10/07/2003	17/07/2003	12.6	6.4	679*	3.96*	-

*Table detailing haematological data for samples included in PrP^c analysis from sCJD, vCJD, and neurological control groups. Table shows date sample taken for haematology analysis (Date 1), the date sample was taken for PrP^c analysis (Date 2). Data for haemoglobin (Hb), white blood cell counts (WBC), platelet count (Plts), red blood cell counts (RBC), and mean cell volume (MCV) are shown. Any abnormalities outside normal ranges are denoted by *, - where no data available.*

Our findings indicate that a reduction in concentration of whole blood PrP^c may be common in vCJD and other neurological diseases but not sCJD. An elevated level of plasma PrP^c may be common in sCJD. These differences between groups appear genuine, presumably reflecting differences in the disease process in the patients or their typical situation in these particular groups and not an artefact of age, specimen collection, storage or analysis. More samples are required for analysis to ensure that differences remain significant. Despite the significance of differences between groups the variation in values are large and there is considerable overlap between CJD groups and control groups, which rules out the exploitation of these differences in whole blood and plasma in screening strategies. These studies expose the limitations in the use of blood PrP^c levels as a diagnostic tool. However they illustrate important observations on the distribution of PrP^c in the peripheral blood of CJD patients and the potential of DELFIA-based PrP assays in clinical practice. The analytical sensitivity of DELFIA-based assays used here represent a significant step towards the development of DELFIA for the detection of PrP^{Sc}, which is a much more reliable indicator of infection, and the current study emphasizes the need to develop assays for its detection in blood [21,152].

Chapter 4: Use of PK and GdnHCL to discriminate between PrP^{c} and PrP^{Sc}

4.1 Introduction

In order to adapt the DELFIA PrP^{c} sandwich assay system used and standardised in the previous chapter for the detection of the disease associated isoform PrP^{Sc} it is necessary to introduce sample processing capable of either distinguishing PrP^{Sc} from PrP^{c} or removing PrP^{c}. As discussed earlier PrP^{Sc} has the same amino-acid sequence as PrP^{c}, and the two isoforms differ only in terms of conformation, their solubility in non-ionic detergents, chaotropic salts, and their resistance to proteolysis by PK. Therefore any anti-PrP antibody used as capture antibody will bind to both PrP^{c} and PrP^{Sc} and there will therefore be competition between the two conformers for binding. In the absence of a conformational specific antibody for either isoform or ligands which interact uniquely with one of the isoforms allowing their separation, it is necessary to develop methods for distinguishing the two isoforms based on exploitation of physical and chemical properties. One defining property of PrP^{Sc} is its partial resistance to proteolysis by PK as defined by Prusiner et al [2]. This property has been exploited in western blot and immunohistochemical methods for the detection of PrP^{Sc} in the CNS and peripheral lympohoreticular tissues, and a common term for PrP^{Sc} has been PrP^{res} referring to the PK resistant portion of the protein. Although the use of PK at a concentration of 50 μG/mL is suitable for the removal of all PrP^{c} for tissue analysis it is likely that conditions used to eradicate PrP^{c} in peripheral blood would be different. This illustrates the inherent problem of using PK as a means of distinguishing between isoforms. The amount of PK required to remove PrP^{c} from any given tissue or fluid is dependent upon the relative amounts of

PrP^c present. Although PrP^{Sc} is partially resistant to protease action it can be degraded completely in high concentrations of PK, and several groups allude to the existence of PK sensitive disease associated PrP isoforms which would be degraded with the use of PK [157]. Despite these drawbacks PK remains one of the few means of distinguishing isoforms from one another to allow the detection of PrP^{Sc}.

For these reasons, in a series of three experiments healthy adult whole blood was treated with range of PK concentrations. PrP^c concentrations were measured by DELFIA to assess the level of proteolysis and the suitability of using PK to eradicate PrP^c in whole blood samples.

4.2 Removal of whole blood PrP^c by PK

Citrate anti-coagulated whole blood was obtained from a healthy adult blood donor, and 100 µL aliquots were treated with 50 µL of a range of PK concentrations between 0 and 500 µG/mL in DH_2O for one hour in a heat block preheated to 37° C. Replicate aliquots without PK were also heated in the same manner to ensure results were comparable. Following incubation 20 µL of 100 mM Pefablock SC (Roche, Switzerland), a protease inhibitor, was added to all samples. Samples were then diluted to 1 mL final volume in DELFIA assay buffer (a 1/10 dilution of whole blood, pefabloc present at 2 mM final concentration) and assayed twice in duplicate by DELFIA for PrP^c. The mean PrP^c concentrations in U/mL were calculated and the standard deviation and concentrations of PrP^c in U/mL were tabulated along with a percentage measurement of PrP^c present after PK action as a factor of that present in the absence of PK. Results from the first assay show that a 0-50 µG/mL range

was sufficient to reduce PrP^c levels in whole blood to 27.55 % of the starting concentration.

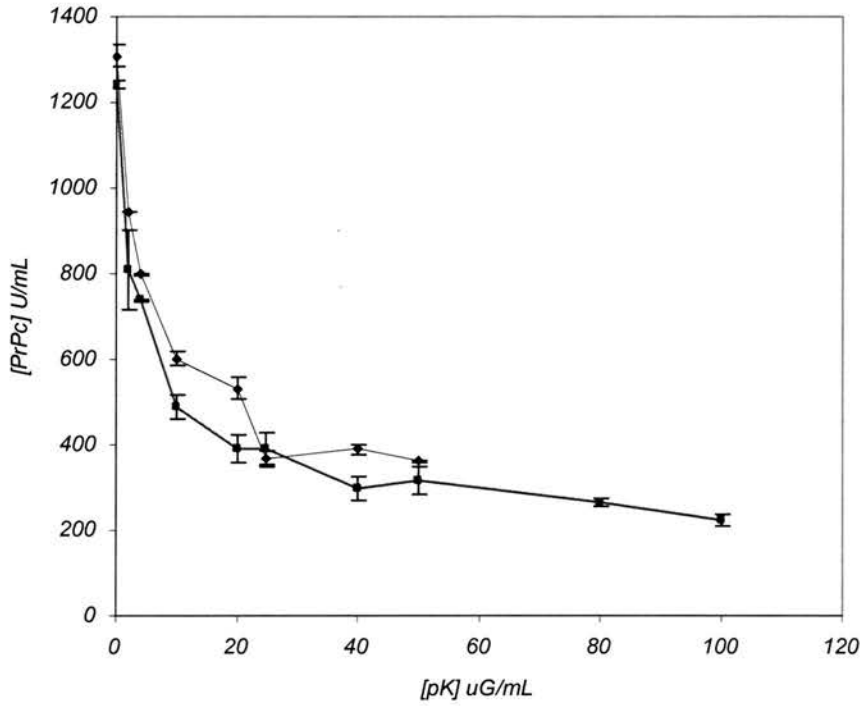
Table 18

[PK] µG/ML	Titre 1	Titre 2	Mean [PrP^c] U/mL	2 SD	% of untreated PrP^c
0	1291	1326	1308.5	49.50	100.00
2	943	944	943.5	1.42	72.11
4	801	798	799.5	4.24	61.10
10	589	614	601.5	35.36	45.97
20	516	549	532.5	46.66	40.70
25	354	382	368.0	39.60	28.12
40	379	398	388.5	26.88	29.69
50	359	362	360.5	4.24	27.55

Results table showing the level of PrP^c detectable after treatment with a range of concentrations of PK (µG/mL). The mean concentration of PrP^c of two titres is shown in U/mL along with ± 2 sd, and % value for reduction of PrP^c from that present in absence of PK

The experiment was repeated with the range of PK concentrations extended to 100 µG/mL. The increased PK concentration was sufficient to reduce starting concentrations further as illustrated in Figure 21 but did not reduce levels completely to background but to 17.95 % of levels present in the absence of PK which represents 223 U/mL PrP^c against a background for blank measurement of 0.27 U/mL.

Figure 21



Graph shows titration curve of the effects of PK treatment [µG/mL] upon the detection of PrP^c [U/mL] in whole blood by DELFIA in two experiments where ranges of 0-50, and 0-100 µG/mL PK were used.

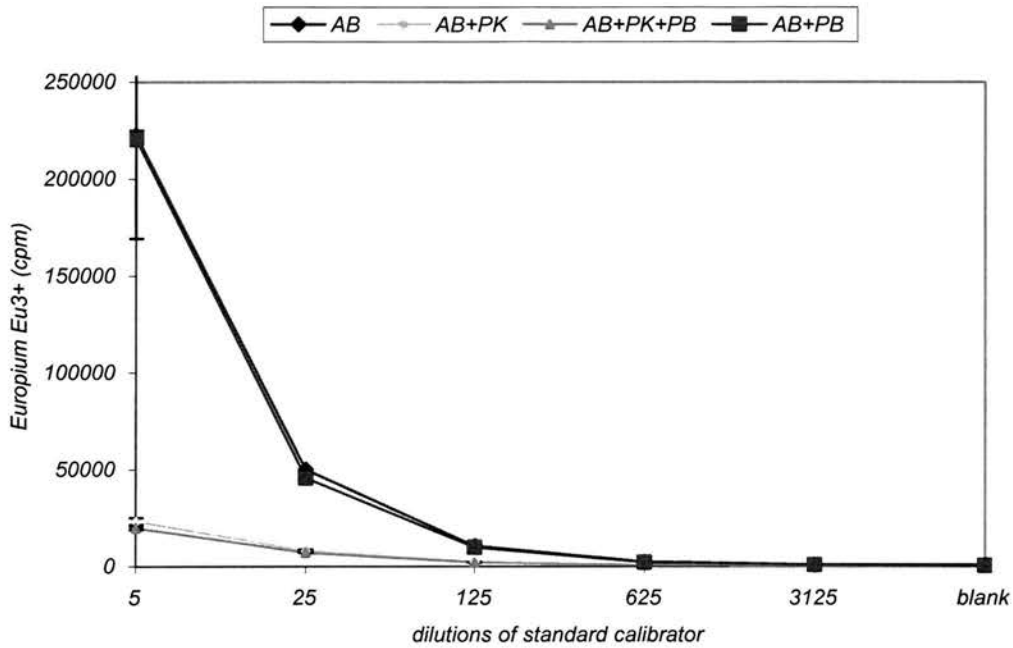
In a third experiment PK concentrations were increased to a range from 0-500 µG/mL. There was concern that the high concentrations of PK required to reduce PrP^c levels in whole blood may interfere with the assay. Evidence of signal disruption by PK and Pefabloc had been inferred (personal communications I. MacGregor). Controls were included in the assay to monitor the effects of Pefabloc. To 100 µL whole blood aliquots 50 µL of DH_2O was added to replace PK in addition to either 20 µL 100mM Pefabloc or DH_2O. Given that PK reduces the detection of PrP^c and is thought to interfere with the assay this is a relatively difficult parameter to measure since its proteolytic action is reducing PrP^c levels whilst its inclusion in

the assay may also be causing a reduction in signal. Provided the same concentrations are added to all samples any effects will be shared. Treatment with 500 μG/mL PK was sufficient to reduce PrP^c detection by 99.86 % to 1 U/mL. This equates to a final PK concentration of 25 μG/mL or 5 μG in each microtitre well. No major difference in signal was found between non-PK treated samples treated with and without Pefabloc; the mean calculated concentrations for PrP^c in these samples were 722 U/mL and 707 U/mL respectively.

4.3 Investigation into inhibitory effects of PK and Pefabloc

In order to consider potential assay disruption by PK or Pefabloc an experiment was designed in which rows of an FH11 precoated plate were incubated for one hour with DELFIA assay buffer alone or supplemented with one or both of PK and Pefabloc at final concentrations of 25 μG/mL or 2mM respectively. After washing, each row was incubated with blanks and dilutions of standard calibrator and the DELFIA protocol for the detection of PrP^c was followed. The means europium counts per minute (cpm) and two times standard deviation for each dilution of the standard calibrator were calculated for each row and plotted to study effects of the different pre-incubations on the detection of PrP^c. As illustrated in Figure 22 below, pre-incubation with assay buffer alone or with 2mM Pefabloc give almost identical europium counts at each dilution of standard calibrator with cpm of ~225000 for the 1/5 dilution.

Figure 22



The graph plots the europium counts in counts per minute (cpm) for the detection of PrP^c in each dilution of platelet standard calibrator. The legend denotes the data series produced from those rows preincubated with assay buffer (AB), PK (PK), pefabloc (PB) or combinations thereof. ± 2 sd *is shown as error bars.*

Supplementing the assay buffer with PK either in the presence or absence of 2mM Pefabloc had a significant effect upon the detection of PrP^c in each dilution of the standard. The detected PrP^c expressed in cpm of the 1/5 dilution of standard reagent was reduced by 89.54 % when assay buffer was supplemented with 25mM PK, and reduced by 91.08 % when both PK and Pefabloc were added to the assay buffer. It is clear that exposure of FH11 coated plate to PK is having an inhibitory effect upon antibody-antigen interactions limiting the capture of PrP^c by FH11. It seems likely that the proteolytic activity of PK would cause limited proteolysis of FH11 so reducing the efficacy of antigen capture, or that despite washing some residual proteolytic activity is retained in plate wells causing the proteolysis of PrP^c in

dilutions of the standard reagent during the sample incubation. The addition of Pefabloc at concentrations used in western blotting for halting proteolytic action does not restore the detection of PrP^c to levels obtained when pre-incubating the plate with assay buffer alone, despite it being present at levels in excess of that required to halt proteolysis. Supplementing assay buffer with Pefabloc for pre-incubation shows that its inclusion has negligible effects upon the detection of PrP^c giving a very similar data series to that obtained with assay buffer alone. Therefore the inclusion of Pefabloc in the pre-incubatory buffer alone does not have any inhibitory effects upon antibody-antigen interaction and also its protease inhibitory activity does not appear to reduce the effects of PK. This would indicate that there is proteolytic activity which is not completely inhibited by Pefabloc and is sufficient to reduce the detection of PrP^c in standard reagent either by reducing the affinity of antibody-antigen interactions or by proteolysis of PrP^c by PK remaining in wells after washing. These results highlight one of the major difficulties in using PK in DELFIA and other prospective PrP^{Sc} assays as a means of removing PrP^c; it has a broad specificity and its activity does not appear to be completely eradicated by protease inhibitors. Given that levels of PrP^c are likely to fluctuate between different samples, optimising the use of PK would be difficult with PK concentrations requiring constant readjustment to ensure that conditions for proteolysis were sufficient to remove all PrP^c but did not interfere with the affinity of antigen capture. These finding support those of MacGregor and Barnard (personal communications) who have found similar degradative effects of PK.

4.4 Assessment of assay inhibition by blood cell lysis buffer

It is likely that the high concentration of PK required to completely degrade PrP^c to background can be reduced by disrupting cell membranes either by freeze-thawing or using a cell lysis buffer prior to treatment. As seen in the previous chapter the effects of freeze thawing led to an increase in detectable PrP^c as a result of cell lysis, the disruption of plasma cell membranes and their association with PrP^c. Such effects will make PrP^c more accessible not only to maximise its detection, but its exposure may reduce concentrations of PK required to eradicate PrP^c so reducing inhibitory effects of PK upon assay performance.

A blood cell lysis buffer (BCL) recipe was designed (see materials and methods section 2.8.4) and a series of experiments were carried out firstly to ensure that this reagent did not interfere with the antibody interactions critical to the DELFIA assay. An FH11 coated microtitre plate was washed and rows A to D loaded with 200 µL of assay buffer and rows E to H with a 1/5 dilution of BCL in assay buffer. Plates were incubated for 1 hour at RT, and washed. The plate was effectively split into 2 to assess the effects of pre-incubation with BCL on the detection of PrP^c in freeze thawed and BCL treated or untreated whole blood samples. BCL treatment consisted of supplementing whole blood with an equal volume of BCL buffer incubating for ten minutes before centrifugation and dilution of supernatant in assay buffer. The fact that BCL treated whole blood samples had already been diluted 1/2 was taken into account when making up final dilutions to allow valid comparisons between the different treatments. Untreated or two times freeze thaw treated whole blood samples were diluted 1/5, 1/10, 1/20 in DELFIA assay buffer. All samples were

assayed 200 µL per well in duplicate on both the assay buffer and BCL preincubated sides of the microtitre plate alongside platelet standard calibrator and QC plasma sample dilutions. The mean concentration of PrP^c in U/mL was calculated, as was the standard deviation between three dilutions. The result for each treated whole blood sample is plotted in Figure 23.

Figure 23

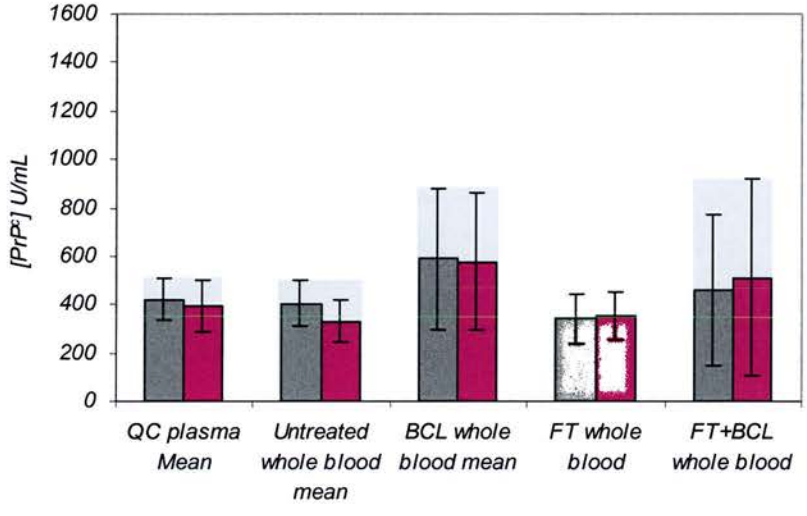


Chart to illustrate the detection of PrP^c (U/mL) by DELFIA in a quality control (QC) plasma sample and whole blood samples from a healthy adult pre-treated by freeze thawing (FT), blood cell lysis buffer (BCL) or a combination of both treatments. Samples assayed on an FH11 coated plate preincubated with assay buffer are shown in grey, and those assayed on the same plate preincubated with BCL buffer at a 1/5 dilution in assay buffer are shown in purple. Error bars show ± 2 sd of measurements at 1/5, 1/10, and 1/20.

The raw data and calculated concentrations do not indicate that the pre-incubation with BCL buffer have any real effect upon the detection of PrP^c compared with pre-incubation with assay buffer alone. However the standard deviations reflect the large differences evident in levels of PrP^c detected in whole blood samples assayed at

different dilutions. The detection of PrP^c increases with dilution probably as a result of matrix quenching effect, this is where there is excess antigen bound to the capture antibody and steric effects prevent interaction of detection antibody paratopes with their epitopes. It appears that whole blood samples require further dilution to ensure better correlation between dilutions. These initial results indicate that BCL buffer treatment ensures the highest detection of PrP^c compared with freeze thawing and combined freeze thaw and BCL treatments, and that its use does not unduly affect assay performance.

This experiment was repeated with whole blood samples assayed at 1/20, 1/40, and 1/80 in order to achieve better correlation, reduce the standard deviation between dilution series, and to reassess the effects of the BCL buffer upon assay performance. The increased dilutions had the desired effect of reducing standard deviations between the dilutions. Comparison between samples assayed on microtitre plates preincubated with BCL buffer and those assayed on the part of the same plate preincubated with assay buffer alone showed a consistent small elevation in concentration of PrP^c detected in all samples assayed on the BCL preincubated area. These findings are illustrated in Figure 24. The effect of increasing the dilution factor therefore reduced variation between concentration measurements at different dilutions. Of all the sample pre-treatments, freeze thawing ensures the highest detection of PrP^c in whole blood samples.

Figure 24

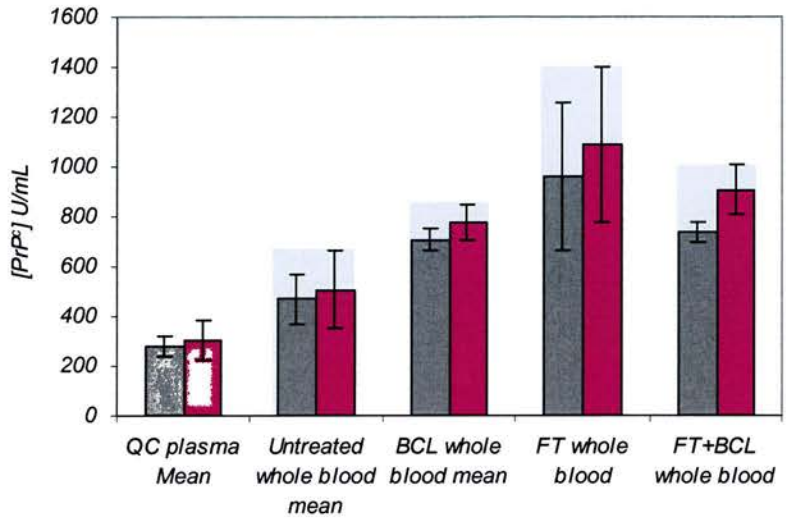


Chart to illustrate the detection of PrP^c (U/mL) by DELFIA in a quality control (QC) plasma sample and whole blood samples from a healthy adult pre-treated by freeze thawing (FT), blood cell lysis buffer (BCL) or a combination of both treatments. Samples assayed on an FH11 coated plate preincubated with assay buffer are shown in grey, and those assayed on the same plate preincubated with BCL buffer at a 1/5 dilution in assay buffer are shown in purple. Error bars show ± 2 sd between measurements at 1/20, 1/40, and 1/80.

This experiment was repeated once again to reconsider the effects of BCL in assay performance and in an attempt to further reduce the standard deviation between dilution series. The assay was carried out exactly as above except that treated whole blood samples were assayed at 1/80, 1/160, and 1/320 dilutions in DELFIA assay buffer. The results shown in Figure 25 below are represented in the same manner as above. The results reconfirm that pre-incubation of part of an FH11 coated plate with BCL buffer at 1/5 dilution in assay buffer causes a small increase in PrP^c concentrations compared with samples assayed on the same plate preincubated with assay buffer alone. This finding is probably a consequence of detergents present in the BCL buffer removing weakly bound FH11 from the microtitre plate. This

ensures that the remaining capture antibody is tightly bound to the microtitre plate and achieves a better affinity with the antigen. Sample preparation by two times snap freeze thawing ensures the highest detection of whole blood PrP^c compared with the use of BCL buffer or a combination of BCL and freeze thaw action.

Figure 25

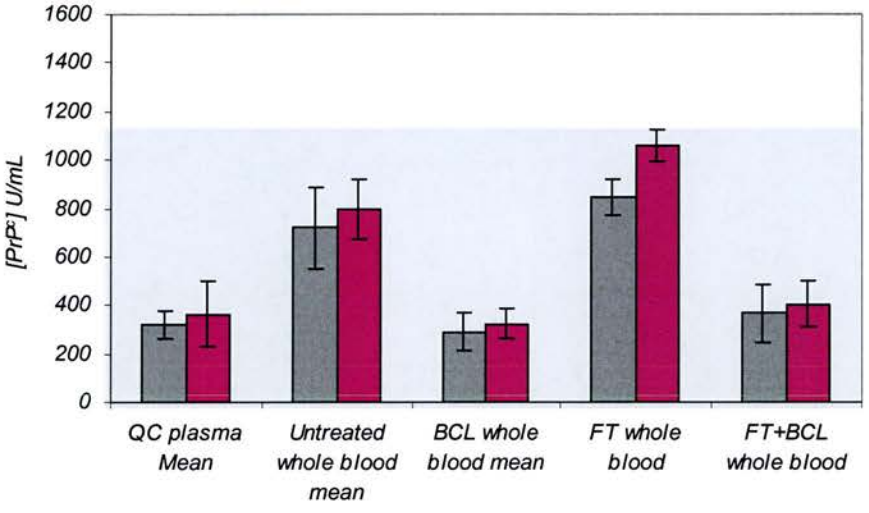


Chart to illustrate the detection of PrP^c (U/mL) by DELFIA in a quality control (QC) plasma sample and whole blood samples from a healthy adult pre-treated by freeze thawing (FT), blood cell lysis buffer (BCL) or a combination of both treatments. Samples assayed on an FH11 coated plate preincubated with assay buffer are shown in grey, and those assayed on the same plate preincubated with BCL buffer at a 1/5 dilution in assay buffer are shown in purple. Error bars show ± 2 sd between measurements at 1/80, 1/160, and 1/320.

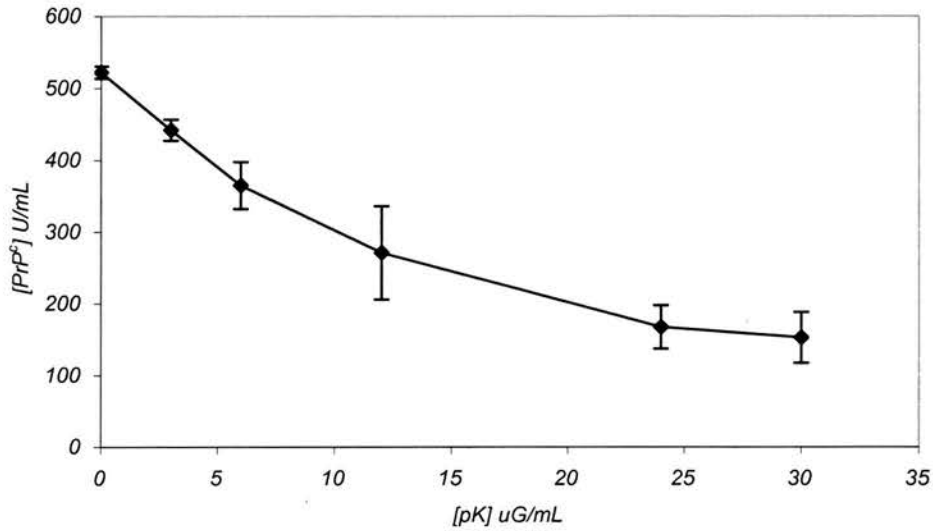
As a result of these findings the use of freeze thawing was adopted as the standard sample preparation procedure prior to assaying separated blood components for PrP^c. Considering samples were routinely stored at -80° Celsius after blood separation this sample treatment was also the most convenient for analysis.

4.5 Titre of PK concentration for the removal of PrP^c from freeze-thaw treated whole blood samples

Continuing studies into exploiting the protease resistance of PrP^{Sc} as a strategy for its detection in peripheral blood, the concentration of PK required to remove PrP^c from freeze-thawed whole blood was assessed. Experiments were set up similarly to those conducted in section 4.2. 50 μL of differing concentrations of PK in DH_2O were added to 100 μL of freeze-thawed whole blood. After incubation for one hour at 37°C, 20 μL of 100 mM Pefabloc was added and 830 μL of assay buffer was added to make a final sample volume of 1 mL. Pefabloc concentrations were always present in an excess to halt proteolysis. Samples were assayed twice in duplicate at 1/100, 1/200, and 1/400 dilutions and the mean concentration of PrP^c and two times the standard deviation were calculated.

Firstly the proteolytic effects of a range of PK concentrations from 0-30 μG/mL (Figure 26) were assessed. PrP^c fell in a dose dependent fashion as concentrations of PK increased, treatment with 30 μG/mL levels decreased PrP^c to 29.26 % of levels detectable in the absence of PK but were not sufficient to reduce levels to background. Treatment of 100 μL of whole blood with 50 μL of 30 μG/mL PK represents exposure of the blood sample to a PK concentration of 10 μG/mL.

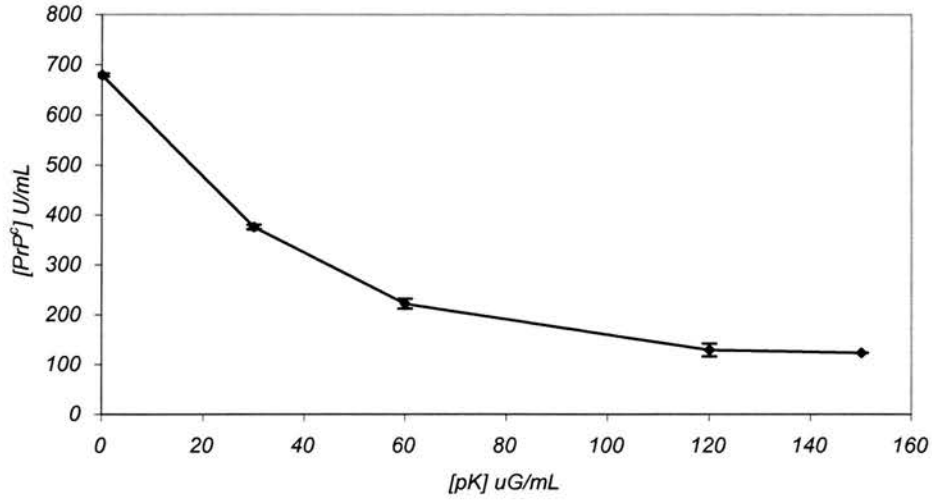
Figure 26



Graph shows titration curve of the effects of increasing concentrations of PK [μG/mL] on the detection of PrP^c [U/mL] in freeze-thawed whole blood by DELFIA. Error bars ± 2 sd.

Consequently PK concentrations were increased three fold to a range between 0 –150 μG/mL which represents a final PK concentration within a range of 0-50 μG/mL. Samples were assayed as above at 1/100, 1/200, and 1/400 dilution. Increasing the concentration of PK led to problems in the detection of PrP^c. As cpm for the higher sample dilutions decreased (as a result of decreased PrP^c detection due to proteolysis) they dropped beneath the level of europium counts obtained for the 3125 dilution of the standard calibrator, their concentration could not therefore be calibrated by the assay since it fell outside the range of the calibrator. This was the case when treating with final concentrations of 40 and 50 μG/mL. The graph in Figure 27 below shows the effects of increasing PK concentrations upon the detection of PrP^c.

Figure 27



Graph shows titration curve of the effects of PK treatment [μG/mL] upon the detection of PrP^c [U/mL] in freeze-thawed whole blood by DELFIA. Error bars represent ± 2 sd

It was therefore difficult to estimate the levels of PrP^c present as a mean value from three dilutions. PrP^c values in U/mL at PK concentrations of 120 and 150 μG/mL were calculated at 1/100 sample dilution only because PrP^c levels in U/mL at 1/200 and 1/400 dilutions could not be measured since europium counts dropped to less than 0.82 U/mL which is the limit of detection of the assay.

In a final experiment 100 μL thawed whole blood aliquots were treated with PK at the following concentrations in 50 μL of DH_2O: 0, 31.25, 62.5, 125, 250, and 500 μG/mL these correspond to the presence of PK at final concentrations in a range from 0 – 166.7 μG/mL. In order to ensure low levels of PrP^c expected after treatment with high concentration of PK were detectable, samples were assayed at a 1/10 dilution. The assay was performed as described above. The results for this assay are summarized in Table 19 below and show the amount of PrP^c detected

following treatment with PK at final concentrations between 0 – 166.7 μG/mL. The use of PK at a final concentration of 42 μG/mL is sufficient to reduce levels of PrP^c to less than 1 % of level of detectable PrP^c in whole blood in the absence of PK. Treatment with PK concentrations of 83.3 and 166.7 μG/mL reduced detectable PrP^c to less than 0.82 U/mL. Therefore a final PK concentration between 42 – 83.3 μG/mL would be sufficient to reduce levels of PrP^c to undetectable levels, see table below.

Table 19

[PK] μG/mL	[PrP^c] U/mL	% Untreated PrP^c levels
0	700.4	100.00
10.5	13.1	1.87
21	12	1.71
42	6.2	0.89
83.3	<0.82	-
166.7	<0.82	-

Table of concentration of PrP^c (U/mL) following treatment with increasing concentrations of PK

This series of assays were beset by problems with PK reducing levels beneath the limit of detection of the assay. Also it was often difficult to ensure accurate PrP^c measurement supported by parallelism between samples dilutions since the dilution factor would reduce the concentration of PrP^c present. There were also many instances where the % CV of sample assayed in duplicate were greater than ten, implying that the presence of PK in assayed samples might be disrupting the capture of PrP^c by monoclonal antibody FH11 as was the case in previous experiments in this chapter which studied the potential inhibitory effects of PK.

There are clear difficulties in using PK as a means of removing PrP^c for the detection of PrP^{Sc} in DELFIA assays. It is difficult to standardise conditions to ensure the

removal of all PrP^{c} given that levels are likely to be different in different samples and different individuals. If PK concentrations are too high they may remove PrP^{Sc} as well as PrP^{c}, and if PK concentrations are too low they will not remove all PrP^{c}. The use of PK will therefore always be technically difficult and limited in sensitivity. Most significant though is that PK would appear to have some degradative effects upon antigen antibody interactions in the DELFIA assay.

4.6 Guanidine hydrochloride differential extraction of freeze-thaw treated healthy adult whole blood samples.

A method has been described by Barnard et al [158] which exploits the solubility of PrP^c in low molar concentrations of chaotropic salt guanidine hydrochloride (GdnHCL) so allowing its separation from disease associated aggregated isoform PrP^{Sc} which remains insoluble at these low molar concentrations but becomes soluble at higher molar concentrations. This method has been used successfully to discriminate between PrP^c and PrP^{Sc} in BSE infected bovine brain tissue. Following differential solubilisation PrP^c and PrP^{Sc} were detected by DELFIA. Under non-denaturing conditions the epitope for the 3F4 antibody is hidden in PrP^{Sc}, but treatment with 6 M GdnHCL denatures the protein increasing its solubility and exposing the epitope ensuring its detection in the DELFIA assay. Here methods were adapted to determine whether this extraction method would be suited to the separation of PrP^c and PrP^{Sc} in whole blood and could be used in the future as a basis for a detection system for PrP^{Sc} in whole blood samples from patients with vCJD.

4.6.1 Isolation of PrP^c in whole blood samples using guanidine hydrochloride

Whole blood samples from healthy adult donors were used to assess PrP^c levels using low molar concentrations of GdnHCL to solubilise the PrP^c present in a sample. To 100 µL whole blood, snap freeze-thawed four times, was added 100 µL 2 Molar GdnHCL in a 1.5 mL eppendorf tube, after a 10 minute incubation 800 µL DELFIA assay buffer was added and the sample was vortexed and spun at 10,000g in an eppendorf centrifuge for 10 minutes at RT. The supernatant was removed for

assay (Sn1) and any pellet or cellular debris was resuspended in 200 μL of 6 Molar GdnHCL and incubated for 10 minutes before the addition of 800 μL of DELFIA assay buffer, vortexing, and centrifugation as above. This supernatant was removed and was assayed (Sn2) (using the standard DELFIA protocol for the detection of PrP^c) along with the Sn1 twice in duplicate at 1/10, 1/100, 1/200, and 1/400 dilutions in assay buffer. Untreated freeze-thaw whole blood supernatants were also assayed twice in duplicate at the same dilutions alongside the platelet standard calibrator reagent and plasma quality control sample. Results are shown in Figure 28.

Figure 28

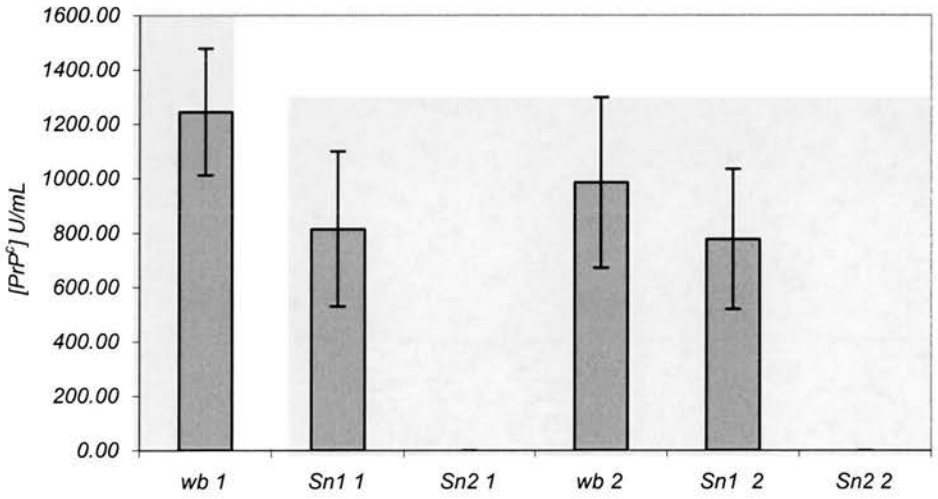


Figure shows the mean concentration of PrP^c U/mL detectable in freeze-thawed whole blood samples (wb1, wb2) and levels detectable in 2 assays of extracted supernatants Sn1 and Sn2. The error bars represent ± 2 sd. Levels of PrP^c detectable in Sn2 supernatants are <0.82 U/mL at the limit of detection of the assay.

Table 20

Sample ID	Mean [PrP^c] U/mL	2 x SD
WB1	1244.67	233.73
Sn1 1	814.58	141.94
Sn2 1	< 0.82	-
WB2	984.9	156.35
Sn1 2	776.8	128.3
Sn2 2	< 0.82	-

Table shows mean values and two times standard deviation (SD) for the detection of PrP^c (U/mL) in untreated freeze-thawed whole blood (WB1, WB2) and in Sn1 and Sn2 supernatants generated by the extraction protocol for each sample. Mean values and $\pm$ 2 sd are calculated from assaying samples at four dilutions as described in the text.

The mean values for detection of PrP^c in untreated freeze-thawed whole blood samples and in extracted supernatants tabulated in Table 20 above show that GdnHCL has been used with some success for the removal of PrP^c from whole blood samples. In the first of two extracted samples the extraction using 1 Molar GdnHCL was able to solubilise 814.58 U/mL PrP^c representing 73.07 % of mean measurement of PrP^c in two untreated whole blood samples (1114.79 U/mL). In the second sample in the Sn1 supernatant 776.8 U/mL PrP^c was detected which represents 69.68 % of mean levels present in untreated whole blood samples. The concentration level of PrP in Sn2 supernatants extracted using 6 Molar GdnHCL is less than the 1/3125 dilution of the assay calibrator at the limit of detection of the assay 0.82 U/mL, so concentrations could not be determined accurately. The low levels of PrP in Sn2 supernatants were expected because the majority of PrP^c would be solubilised in the first extraction with 1 Molar GdnHCL and you would not expect to find disease associated PrP^{Sc} in whole blood samples from healthy adult donors. Differential extraction using GdnHCL can isolate PrP^c from healthy adult whole blood samples. The extraction is not complete; the concentrations of PrP^c present in 1 Molar

supernatants represent approximately 70% of levels in untreated whole blood samples. It is likely that some residual PrP^c remains in the pellets. The performance of the assay appeared unaffected by the presence of GdnHCL, the Coefficient of variation (CV) for sample duplicates remains less than 10 %.

This assay was repeated to ensure results could be replicated and also to investigate the presence of PrP^c in pellets. The assay was set up exactly as before except that the pellets remaining after the extraction with 6 M GdnHCL were resuspended in DELFIA assay buffer and assayed at 1/10, 1/100, 1/200 and 1/400 dilutions. Table 21 shows the mean concentrations of PrP^c detected for untreated whole blood and the Sn1 and Sn2 supernatants and the 1/10 dilution of the resuspended pellet. The two times standard deviation measurements are high, this is a consequence of including data for PrP^c measurement from samples assayed at 1/10 dilution, under these conditions values did not show good parallelism as was the case with concentrations obtained when samples were assayed at higher dilutions, this is due to matrix quenching. The presence of detectable PrP^c in 6 Molar extracted supernatants was very low and levels could not be calibrated since europium counts were below the limit of detection of the assay. There were small amounts of PrP^c detectable in the pellets values shown are those calculated from 1/10 dilution, these values could not be verified with values obtained when samples were assayed more dilute since europium counts dropped below the limit of detection of the assay.

Table 21

Sample ID	Mean [PrP^c] U/mL	2 x SD
WB1	747.5	375.61
Sn1 1	734.73	484.53
Sn2 1	<0.82	-
Pellet 1	20.3	-
WB2	678.23	365.34
Sn1 2	720.75	444.68
Sn2 2	< 0.82	-
Pellet 2	16.5	-

Table shows mean values and two times standard deviation (SD) for the detection of PrP^c (U/mL) in untreated freeze-thawed whole blood (WB1, WB2) and in Sn1 and Sn2 supernatants generated by the extraction protocol for each sample. Mean values and ± 2 sd are calculated from assaying samples at four dilutions as described in the text.

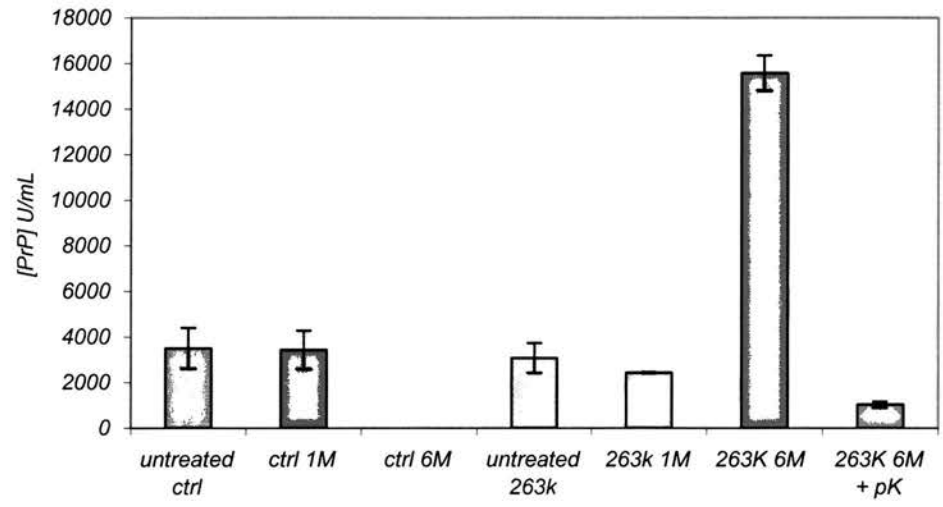
The results from this experiment support the findings of the previous experiment; the majority of PrP^c present in control whole blood samples can be solubilised and extracted using 1 Molar GdnHCL (Sn1). PrP^c is present at undetectable levels in 6 M supernatants (Sn2) and any loss during extraction can be explained by small amounts present in the pellets. The results again verify the suitability of using guanidine hydrochloride as a method for the isolation of PrP^c from whole blood samples. This methodology provides an alternative to PK treatment as a means of removing PrP^c.

4.7 Guanidine Hydrochloride differential extraction of 263K-scrapie infected and uninfected control hamster brain homogenates

The experiments above illustrate the suitability of using GdnHCL to solubilise PrP^c from human whole blood samples. In order to verify the findings of Barnard et al [158] that this method is suitable for the isolation of both PrP^c and PrP^{Sc} from TSE infected brain material the following experiments were performed.

Brain material from Syrian hamsters infected with 263K-scrapie strain and uninfected control healthy animals obtained from the Institute of Animal Health Edinburgh, UK were homogenised to 10 % (w/v) following methods described in the materials and methods section 2.8. Extraction of PrP^{c} and PrP^{Sc} was carried out following methods described by Barnard et al [158]. To 50 μL 10 % brain homogenate was added the same volume of 2 Molar GdnHCL in a 1.5 mL eppendorf tube to give a final concentration of 1 Molar GdnHCL. After vortexing and a 10 minute incubation, 900 μL of DELFIA assay buffer was added and the mixture was spun at 10,000g in an eppendorf centrifuge for 10 minutes at RT. The supernatant was removed (Sn1) and the pellet resuspended in 100 μL of 6 Molar GdnHCL this mixture was vortexed, incubated and spun as before and the supernatant removed (Sn2) for analysis. Untreated scrapie infected and control brain homogenates were assayed alongside the extracted supernatants by DELFIA for PrP, each sample was assayed in doubling dilutions from 1/200 to 1/1600. In order to verify that the extraction method separated PrP^{c} from PrP^{Sc}, supernatants extracted from control and scrapie infected brain homogenate were treated with 50 μG/mL PK prior to analysis. Samples were assayed following the standard DELFIA protocol for PrP^{c}. The mean concentrations of PrP and two times the standard deviation were measured and results are shown in the Figure 29 below.

Figure 29



Concentrations of PrP detected by DELFIA in control healthy hamster brain homogenate and in 263k-scrapie infected hamster brain homogenate. PrP concentrations in untreated samples and in 1 and 6 Molar guanidine hydrochloride extracted supernatants and in PK treated 6 Molar supernatant from 263k scrapie sample are plotted, ± 2 sd is shown as error bars. PrP was undetected after PK treatment in both supernatants from control brain material and in 1 Molar supernatant from 263k infected brain material (data not shown).

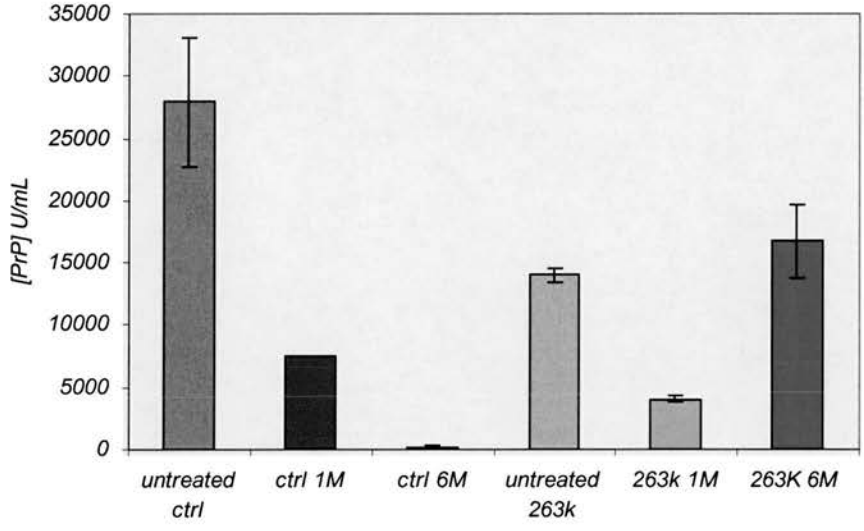
The results verify that this method can be used successfully to distinguish scrapie infected hamster brain from uninfected control hamster brain given the presence of detectable PrP in 6 Molar GdnHCL extracted supernatant. The aggregated nature and the conformational differences characteristic of PrP^{Sc} make it less soluble in low molarity chaotropic salt than non-aggregated normal PrP^{c}, so allowing separation. In an analysis of healthy hamster brain homogenate 97.97 % of PrP^{c} detected in untreated brain homogenate was successfully extracted and detected in the 1 Molar GdnHCL supernatant, there was no detectable PrP in the 6 Molar GdnHCL extracted supernatant. PrP was absent in PK treated supernatants extracted from healthy hamster brain homogenate. In assays of scrapie infected hamster brain homogenates 79.14 % of PrP^{c} detected in DELFIA analysis of untreated homogenate was

successfully extracted and detected in the 1 Molar GdnHCL supernatant. Analysis of the 6 Molar GdnHCL extracted supernatant found extremely high levels of PrP (15,580.5 U/mL). Such high levels of PrP are not detectable when the scrapie brain homogenate is assayed untreated because the epitope for the detection antibody 3F4 is occluded in the altered conformation of aggregated PrP^{Sc}. It is only after treatment with 6 Molar GdnHCL that the PrP^{Sc} is unfolded and solubilised, and the 3F4 epitope is no longer hidden and is exposed for binding. This finding and the absence of detectable PrP in the 6 Molar GdnHCL supernatants from uninfected healthy hamster brain argues that the PrP in the 6 Molar GdnHCL supernatant is the disease associated isoform PrP^{Sc}. Indeed PrP remains detectable only in the 6 Molar GdnHCL supernatant from scrapie infected brain homogenate after treatment with PK. This is to be expected since by definition PrP^{Sc} is partially resistant to proteolysis with PK. Levels of PrP are reduced significantly by PK treatment, probably a consequence of treating PrP^{Sc} with PK in the presence of GdnHCL. When denatured and solubilised PrP^{Sc} is removed from its aggregated state and so adopts conformations which like PrP^{c} are more susceptible to proteolysis with PK. In addition the FH11 capture antibody has two N-terminus epitopes one of which is N-terminal to sites of PK cleavage, the loss of which would contribute to the reduced detection of PK treated PrP.

The differential extraction of 263K-scrapie infected hamster brain homogenate and with brain homogenate from a healthy uninfected hamster was repeated to ensure reproducibility of the technique. Extracted supernatants were assayed twice in duplicate at a 1/20 dilution of the original brain homogenate, untreated samples were

assayed at 1/100, 1/200, and 1/400. This produced similar results and they are illustrated in Figure 30 below. Again there is very little PrP detectable in the 6 M GdnHCL supernatant of control healthy hamster brain, but very high levels of PrP detected in the same supernatant from the scrapie infected brain homogenate.

Figure 30



Concentrations of PrP detected by DELFIA in control healthy hamster brain homogenate and in 263k-scrapie infected hamster brain homogenate. PrP concentrations in untreated samples and in 1 and 6 Molar guanidine hydrochloride extracted supernatants are plotted, error bars show ± 2 sd.

The main difference from the previous experiment is that the concentrations of PrP detected in untreated brain homogenates are much higher than levels of PrP present in the 1 M GdnHCL supernatants whereas in the previous experiment they were very close as would expected. This finding is a direct result of assaying extracted supernatants at neat rather than at a range of doubling dilutions between 1/100 – 1/1600 as in the previous experiment. Assaying of samples where PrP is present at

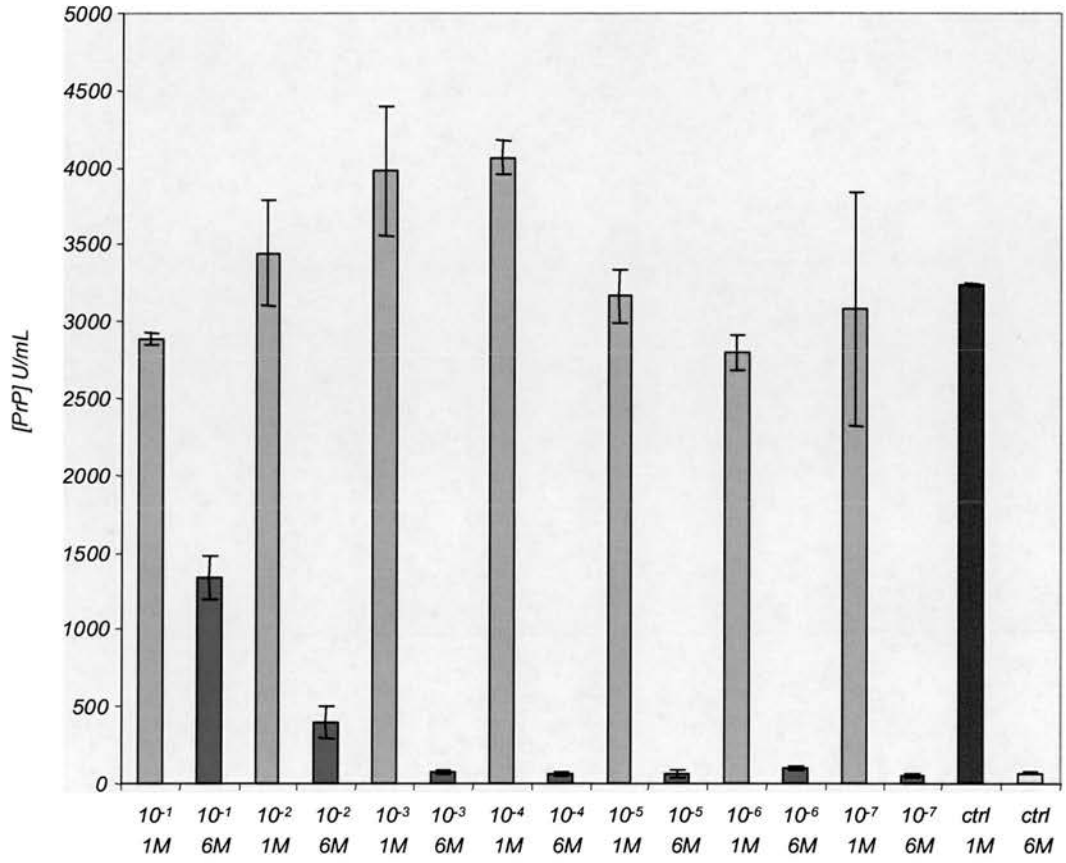
high concentrations gives a poor quantification of actual levels present due to matrix quenching effect.

4.7.1 Sensitivity of differential extraction technique for detection of PrP^{Sc}

To assess the sensitivity of using GdnHCL for the detection of disease associated PrP, 263K scrapie infected hamster 10% brain homogenate was spiked into uninfected healthy control hamster 10 % brain homogenate in serial log dilutions from 1/10 to 1/10,000,000 ($10^{-1} - 10^{-7}$). 50 µL of each dilution and unspiked healthy control hamster brain homogenate was differentially extracted following methods described above and extracted supernatants were assayed twice in duplicate. Mean concentrations of PrP in U/mL were calculated and corrected for the dilution factor. The concentration of PrP detectable in the 1 Molar supernatant is expected to remain relatively constant and the presence of disease associated PrP in the 6 Molar supernatant is expected to decrease with each serial dilution until it approaches background levels. The assaying of healthy uninfected hamster brain homogenate will provide a background threshold or cut-off value for the 6 Molar supernatant, activity in excess of this threshold value would indicate the presence of disease associated PrP^{Sc}. The results for this spiking study are shown in Figure 31 below. Disease associated PrP^{Sc} is readily detectable in 6 Molar supernatants of 10^{-1}, and 10^{-2} log spiked samples in excess of the mean cut-off level of 66 U/mL. In spiked sample dilutions between 10^{-3} and 10^{-7} detectable levels of PrP in 6 Molar supernatants are similar to levels detected in healthy control hamster brain homogenate supernatants and cease to decrease with further dilution. At present this technique has a detection sensitivity of two log dilutions of a 10 % 263K-scrapie

brain homogenate. Infectivity studies of 263K-scrapie infected hamster brain homogenate by bioassay titrate its infectivity to approximately 10^{10} LD_{50}/G [159] since brain homogenates are at 10 % the starting material is at 10^{9} LD_{50}/G. This being the case the detection sensitivity found here of at best 10^{-2} log dilutions expressed in terms of infectivity is 10^{7} LD_{50}/G, equivalent to a 0.5 µL spike.

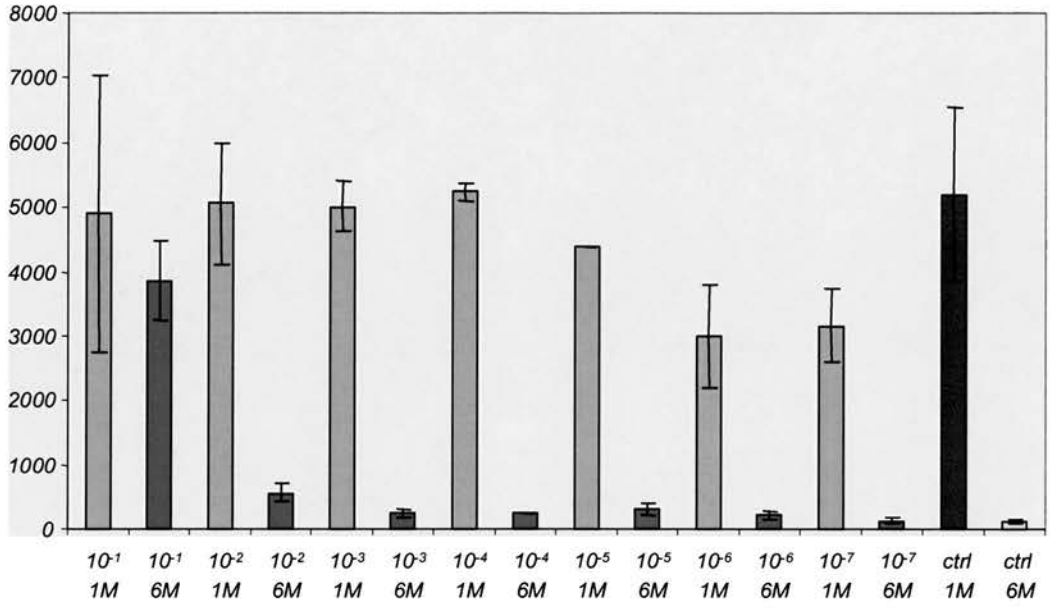
Figure 31



Concentrations of PrP (U/mL) detected in 1(light blue) and 6 Molar (dark blue) guanidine hydrochloride extracted supernatants from healthy control hamster brain homogenates spiked in increasing log dilutions (10-1 –10-7) with 263K-scrapie infected hamster brain homogenate and unspiked controls 1M (green) 6M (yellow). Error bars show ± 2 sd.

This spiking experiment was repeated as described above to verify these results, the findings are illustrated in Figure 32. The reliable limit of detection is 10^{-2} log dilution as in the previous assay. Levels of PrP detected in the 6 Molar supernatants from dilutions 10^{-3} to 10^{-7} are often in excess of the cut-off level of 134 U/mL, but they do not decrease with further dilution and this would indicate that levels have reached the background and it is difficult to determine with any certainty that these levels represent disease associated PrP or background binding. If units of PrP are represented as a concentration the limit of detection for disease associated PrP in 263K-scrapie infected hamster brain homogenate from these two assays is between 10.45 to 14.85 nG/mL based upon assay calibration of one unit equivalent to 26 picograms.

Figure 32



Concentrations of PrP (U/mL) detected in 1 and 6 Molar guanidine hydrochloride extracted supernatants from healthy control hamster brain homogenates spiked in increasing log dilutions (10-1 –10-7) with 263K-scrapie infected hamster brain homogenate. Error bars show ± 2 sd.

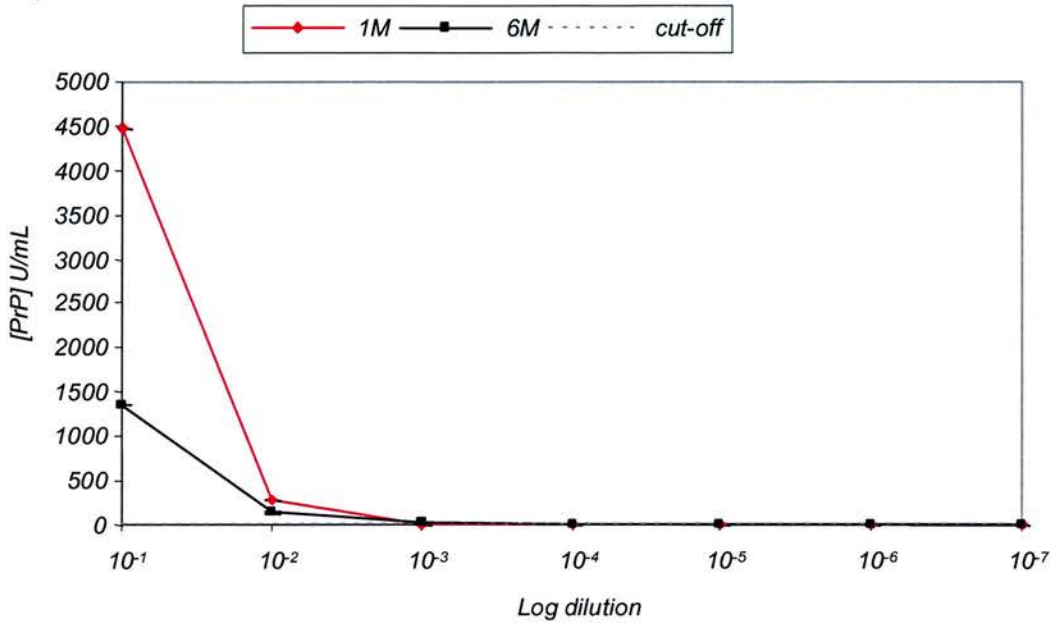
4.7.2 Increases in sensitivity by removal of competing PrP^c in healthy hamster brain homogenate.

Despite separation with GdnHCL, the large quantities of PrP^c present in healthy hamster brain homogenate may be contributing to the presence of total PrP in the 6 Molar supernatants of healthy hamster brain homogenates. It is conceivable that there will be a limit on the quantity of PrP^c able to be solubilised in the 1 Molar GdnHCL, any PrP^c which is not solubilised will be present in the 6 Molar supernatant and will compete with disease associated PrP^{Sc} for binding to capture monoclonal antibody FH11. To assess whether reducing the amount of PrP^c in the diluent buffer would increase the sensitivity of the assay, scrapie infected hamster brain homogenate was spiked into homogenisation buffer instead of into a diluent of healthy hamster brain homogenate. As in the spiking assays described above, buffer spiked in a range dilutions between 10^{-1} and 10^{-7} logs was differentially extracted along with both homogenisation buffer and scrapie infected brain homogenate. Extracted supernatants were assayed twice in duplicate by DELFIA.

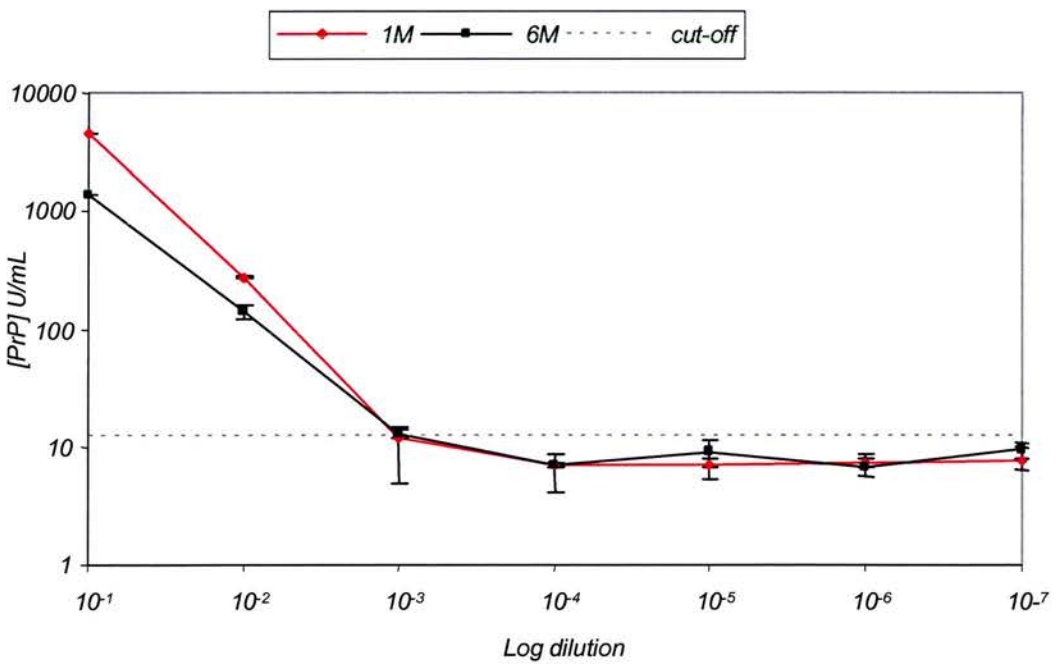
Results are illustrated in Figure 33. Disease associated PrP^{Sc} in 263K-scrapie infected hamster brain homogenate can be detected to 10^{-3} above a cut-off provided by assaying the presence of signal in the 6 Molar supernatant of homogenisation buffer. It is in fact easier therefore to detect disease associated PrP in the absence of large amounts of competing PrP^c from healthy hamster brain homogenate.

Figure 33

A)



B)



The presence of PrP in 1 & 6 Molar GdnHCL supernatants in homogenisation buffer spiked in log dilutions with 263K-scrapie infected hamster brain homogenate are shown in red and black. Error bars represent ± 2 sd. The dotted line represents the cut-off of the level of detectable PrP ± 2 sd in the 6 Molar supernatant of homogenisation buffer a signal above which indicates the presence of disease associated PrP. Graph A) shows a normal scale and graph B) a logarithmic scale on the y-axis.

4.8 Differential extraction of human plasma samples spiked with vCJD brain homogenate.

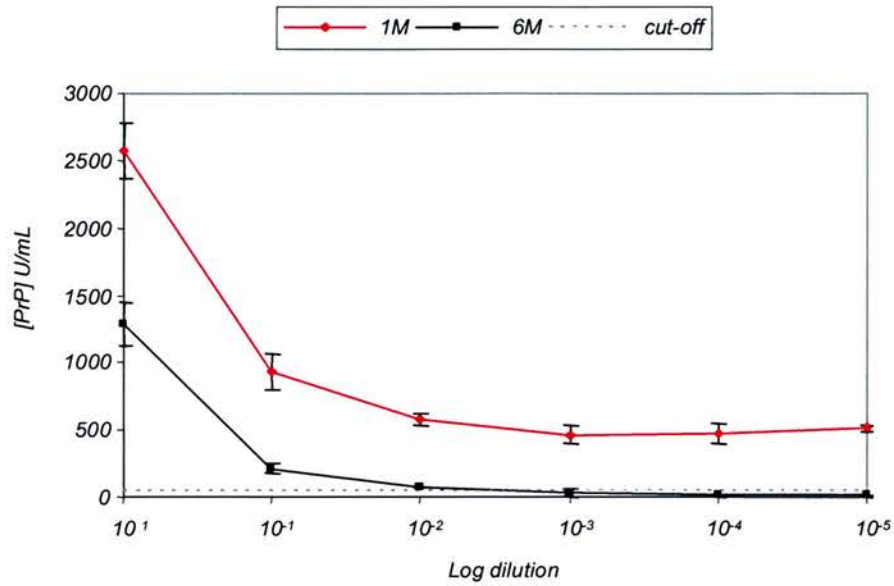
Guanidine hydrochloride has been used successfully to compartmentalise PrP^{c} from whole blood samples from healthy human adult blood donors, and to isolate disease associated PrP^{Sc} from 263k-scrapie infected hamster brain homogenate distinguishing it from healthy control hamster brain. To ensure that this approach would be a suitable for the isolation and detection of disease associated PrP in humans with vCJD, brain homogenate from a patient with neuropathologically confirmed vCJD was spiked into platelet poor plasma prepared from whole blood samples from healthy adult blood donors and assayed as above. Ideally tests designed for the detection of disease associated PrP^{Sc} in human peripheral blood from vCJD patients would be developed using blood samples from these patients. However vCJD clinical blood samples exist in extremely small numbers and in small volumes. Since PrP^{Sc} is likely to be present in very low concentrations in peripheral blood it is only likely to be detected by the most sensitive assays. It is sensible therefore to retain these samples for assay validation and develop the sensitivity of prospective assays using vCJD brain material and peripheral tissues as a source of PrP^{Sc}. Spiking of these into control peripheral blood is an attempt to mimic the presence of PrP^{Sc} in peripheral blood as it supposedly exists in individuals infected with vCJD.

Platelet poor plasma was prepared by spinning healthy adult whole blood at 3000g for 30 minutes and removing the supernatant. 10 % vCJD brain homogenate and 10 % brain homogenate from a patient who died of Alzheimer's disease as non-CJD control were prepared from brains supplied by the National CJD Surveillance Unit,

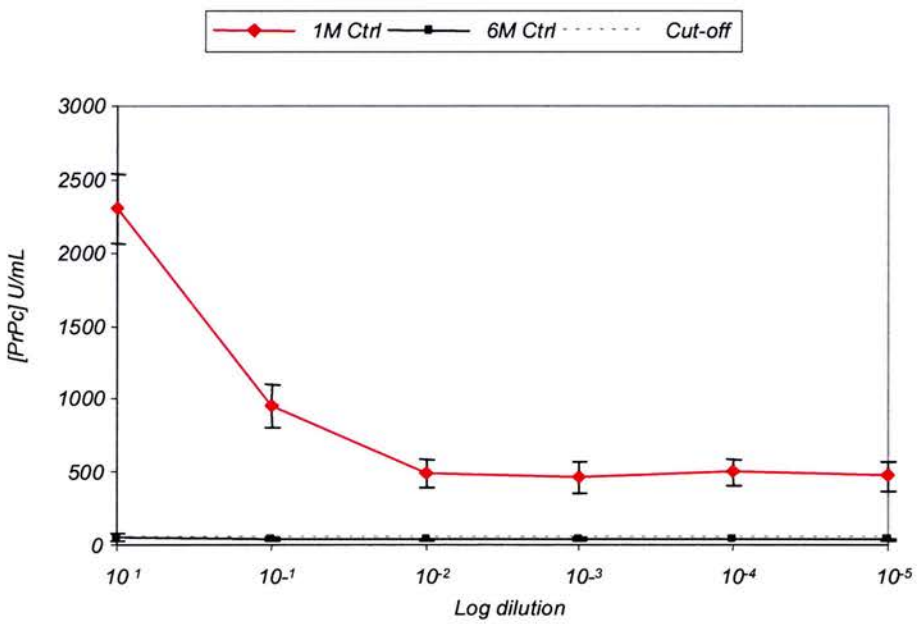
Edinburgh, UK to the National Institute of Biological Standards and Controls for use as reference standards. vCJD brain homogenate was spiked into platelet poor plasma in log dilutions in a range between 10^{-1} and 10^{-5}. 100 μL of spiked plasma, unspiked plasma, or 10 % brain control or vCJD brain homogenate was differentially extracted as before. The mean concentrations of PrP detected in extracted supernatants and two times the standard deviation were calculated and data was plotted for each spiked sample and control to establish the sensitivity of this technique for the detection of disease associated PrP in vCJD brain homogenate spiked into human plasma. The results are shown in Figure 34.

Figure 34

A)



B)



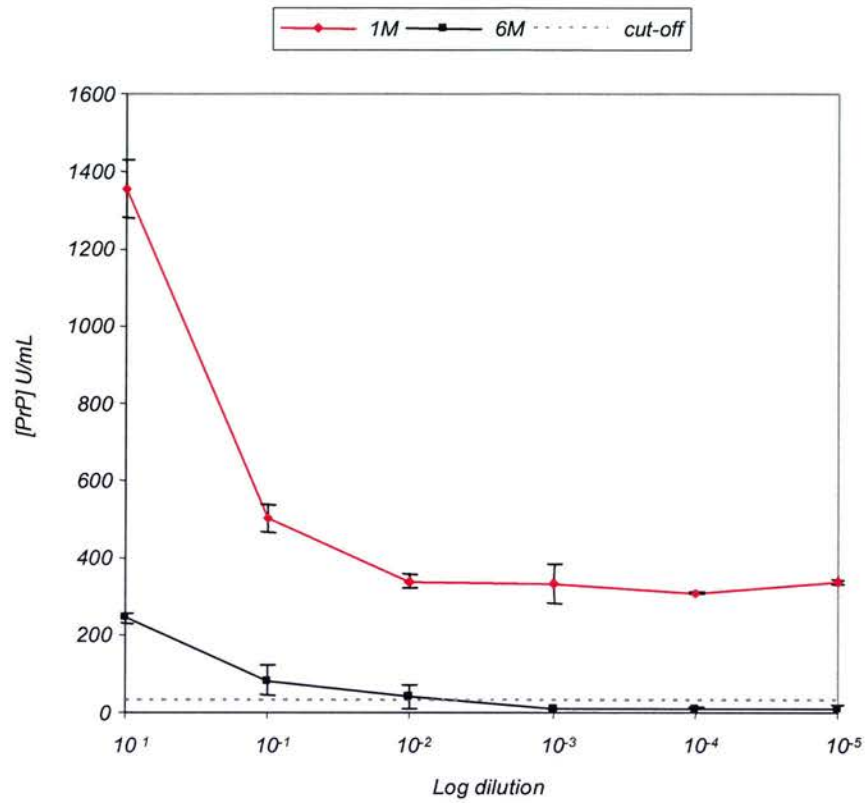
The presence of PrP in 1 & 6 Molar GdnHCL supernatants in platelet poor plasma spiked in log dilutions with vCJD (A) and control Alzheimer's (B) 10% brain homogenate are shown. Error bars represent two times the standard deviation. The dotted line represents the cut-off or threshold level of PrP detected in the 6 Molar supernatant of control platelet poor plasma ± 2 sd.

Using the mean concentration plus two times the standard deviation of the concentration of PrP in the 6 Molar supernatant extracted from platelet poor plasma from a healthy adult donor as a cut-off, vCJD spiked material can be detected to 10^{-2} dilution, a 1/100 dilution of 10 % vCJD brain homogenate. As the presence of the spike becomes more dilute the level in the 6 Molar supernatant drops beneath the cut-off. Levels of PrP detected in the 1 Molar supernatant drop as the contribution of PrP^{c} from the spike becomes less and the detected level reaches that of unspiked platelet poor plasma. This sensitivity for the detection of disease associated PrP in spiked plasma samples is the same as that seen in spiking experiments with 263K-scrapie infected brain homogenate. This experiment verifies the suitability of the use of GdnHCL and DELFIA assay approach for the detection of disease associated PrP in samples from patients with vCJD spiked into platelet poor plasma. PrP in the 6 Molar extracted supernatant from plasma samples spiked with control Alzheimer's brain homogenate is not detectable above cut-off. The disease associated PrP in vCJD brain homogenate may not be physiologically similar to that which you might expect to find in the peripheral blood of patients with vCJD, however in the absence of clear evidence that a disease associated form of PrP in peripheral blood is different from PrP^{Sc} detected in the central nervous tissue, and in the absence of a positive blood control this is the best and only alternative for the development of these types of assays.

This assay was repeated to verify the above result using 10 % vCJD brain homogenate supernatant as a spike rather than whole brain homogenate. As the Figure 35 illustrates this change to the spike affects the concentrations of PrP detected in 6 Molar supernatants but not the limit of detection which remains

unaffected at 10^{-2} log dilutions or 10^{-3} of wet vCJD brain homogenate spiked into platelet poor plasma, see Figure 35 below.

Figure 35



The presence of PrP in 1 & 6 Molar GdnHCL supernatants in platelet poor plasma spiked in log dilutions with vCJD 10% brain homogenate supernatants are shown in red and black. Error bars represent two times the standard deviation. The dotted line represents the cut-off or threshold level of PrP detected in the 6 Molar supernatant of control platelet poor plasma ± 2 sd.

4.9 *Use of guanidine hydrochloride in a conformation dependent immunoassay (CDI) for the detection of PrP^{Sc} in vCJD brain spiked plasma samples.*

The materials and methods section 2.4 describes an experimental approach for the detection of abnormally folded disease associated PrP^{Sc} isoform, based upon exposure of the 3F4 monoclonal antibody epitope by the presence of the chaotropic salt GdnHCL. CDI techniques developed by Safar et al [21] were used here to assess the suitability of this approach for the detection of PrP^{Sc} in vCJD brain homogenates spiked into platelet poor plasma. Platelet poor plasma prepared from a healthy adult donor was spiked with 10 % brain homogenate in a range of log dilutions between 10^{-1} to 10^{-5} in 0.5 log increments. Native and denatured samples from the dilution series along with native and denatured samples from six platelet poor plasma controls were assayed twice in duplicate by DELFIA for PrP following the methods described in the materials and methods section 2.4. The layout of samples assayed is shown in Figure 36 below. Here we are attempting to detect small quantities of PrP^{Sc} in the presence of substantial amounts of PrP^{c}. Because the capture antibody binds both PrP^{c} and PrP^{Sc} any detectable signal increase due to PrP^{Sc} above the substantial level of PrP^{c} is likely to be small.

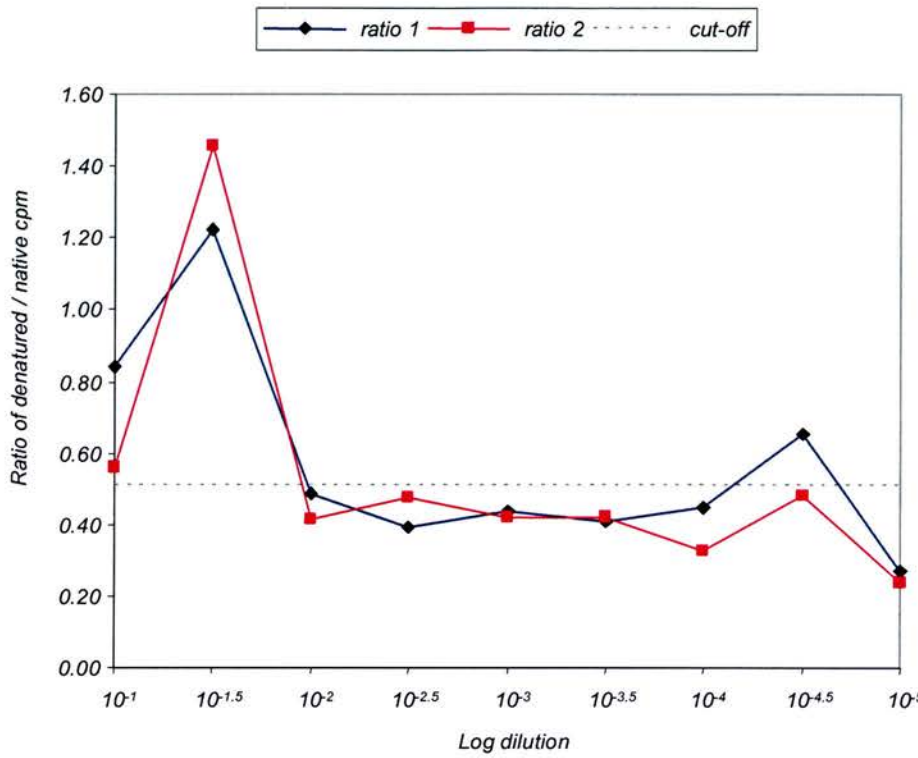
Figure 36

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	BLANK	$10^{-1.0}$	Native	$10^{-1.0}$	Denat	$10^{-1.5}$	Native	$10^{-1.5}$	Denat	$10^{-2.0}$	Native
B	$10^{-2.0}$	Denat	$10^{-2.5}$	Native	$10^{-2.5}$	Denat	$10^{-3.0}$	Native	$10^{-3.0}$	Denat	$10^{-3.5}$	Native
C	$10^{-3.5}$	Denat	$10^{-4.0}$	Native	$10^{-4.0}$	Denat	$10^{-4.5}$	Native	$10^{-4.5}$	Denat	$10^{-5.0}$	Native
D	$10^{-5.0}$	Denat	BLANK	BLANK	$10^{-1.0}$	Native	$10^{-1.0}$	Denat	$10^{-1.5}$	Native	$10^{-1.5}$	Denat
E	$10^{-2.0}$	Native	$10^{-2.0}$	Denat	$10^{-2.5}$	Native	$10^{-2.5}$	Denat	$10^{-3.0}$	Native	$10^{-3.0}$	Denat
F	$10^{-3.5}$	Native	$10^{-3.5}$	Denat	$10^{-4.0}$	Native	$10^{-4.0}$	Denat	$10^{-4.5}$	Native	$10^{-4.5}$	Denat
G	$10^{-5.0}$	Native	$10^{-5.0}$	Denat	PPP1	Native	PPP1	Denat	PPP2	Native	PPP2	Denat
H	PPP3	Native	PPP3	Denat	PPP4	Native	PPP4	Denat	PPP5	Native	PPP5	Denat

Native and denatured portions of each spiked sample are assayed twice in duplicate alongside five platelet poor plasma samples (PPP) that are assayed to provide the cut-off value above which the presence of abnormal PrP^{Sc} is detectable.

The mean europium counts for each sample duplicate were calculated and the means of the denatured sample were divided by the means of the native sample to provide a ratio for each spiked dilution and control. The mean ratio plus two standard deviation of the six platelet poor plasma samples was calculated and used as a cut-off for the presence of disease associated PrP, given that you would not expect to find this in plasma samples selected at random from healthy adult blood donor controls. The ratios for each of the two dilutions series are plotted in Figure 37 below.

Figure 37



The mean ratios of denatured / native cpm for two log dilution series (ratio 1, 2) of platelet poor plasma samples spiked with 10 % vCJD brain homogenate. The cut-off level is the mean ± 3 sd for five platelet poor plasma samples from healthy adults.

The results from this conformation dependent immunoassay (CDI) are not what were expected. Given that there is no abnormally folded aggregated PrP^{Sc} present in control plasma samples you would expect similar levels of detection in both native and denatured samples producing a ratio of one or slightly above one considering that denaturation and unfolding of PrP^{c} to a monomer may afford a slightly better interaction with the 3F4 monoclonal antibody than native PrP^{c}. However in this assay counts decrease in denatured GdnHCL treated samples compared to the untreated native samples so producing ratios less than one. Analysis of data produced from the two runs of the spiked plasma samples also show less binding in

the denatured sample than with the native sample, apart from the $10^{-1.5}$ log dilution. This is not what was expected since the vCJD spiked samples contain PrP^{Sc} and denaturation with GdnHCL should expose the 3F4 epitope which remains hidden in the native sample thereby giving a ratio in excess of one. One possibility is that the presence of GdnHCL which is at a final concentration on the microtitre plate of 0.4 Molar may be interfering with the capture of antigen by MAb FH11, however since GdnHCL is added to native samples at the same final concentration the same degree of inhibition would also be evident in native samples. More plausible explanations are that denaturation and unfolding of PrP reduces the affinity of the interaction between FH11 and PrP so producing a lower detection in denatured samples than in native samples, the antibody pairing of MAbs 3F4 and FH11 is effective for the detection of denatured PrP, or that denaturation accelerates the breakdown of PrP. Despite these unexpected findings, the presence of abnormal disease associated PrP^{Sc} can be detected to $10^{-1.5}$ log dilutions of 10% vCJD brain homogenate when spiked into plasma above a control cut-off using FH11 and 3F4 monoclonal antibodies. The use of different anti-PrP monoclonal antibodies as capture may prove more suitable for a more consistent capture of native and denatured PrP so producing higher ratios which may increase assay sensitivity.

Chapter 5 Use of CDI for detection of PrP^{Sc}: a comparison of different monoclonal antibody pairs and their effects upon assay sensitivity.

5.1 Introduction

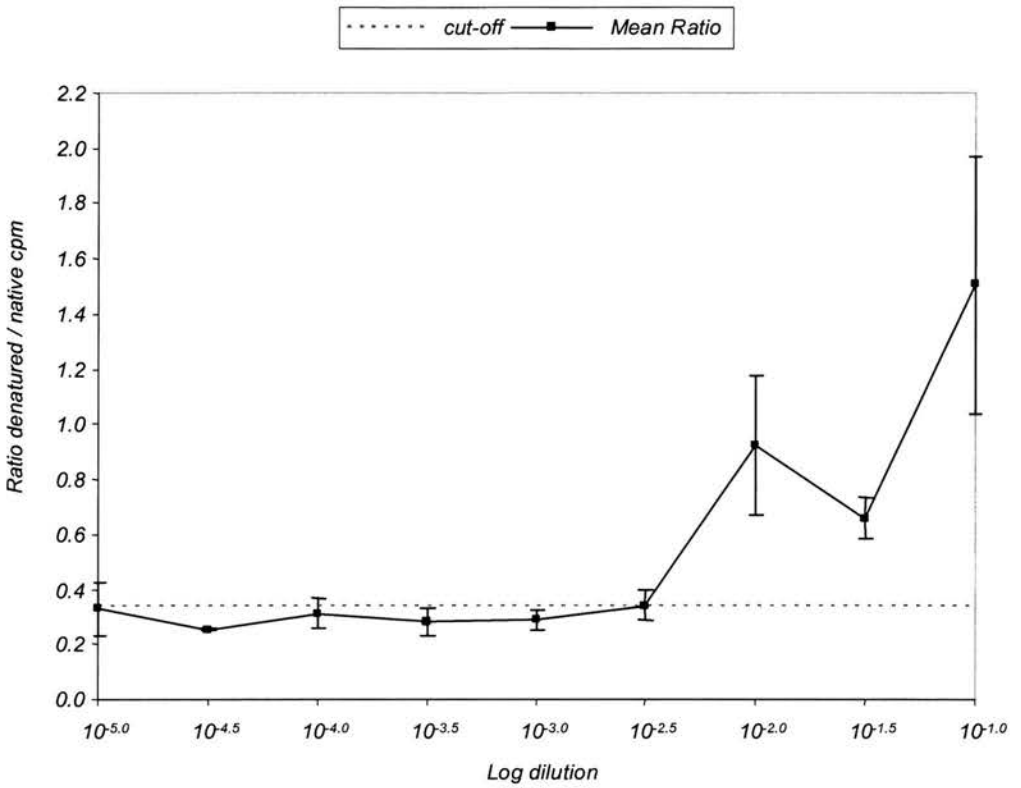
The conformation dependent immunoassay described in section 4.9 requires considerable improvements in sensitivity for the detection of PrP^{Sc} in brain tissue from patients with vCJD before it can be considered as a possible means for the sensitive detection of PrP^{Sc} in the peripheral blood of patients with vCJD. Capture and detection antibodies that have a high affinity for PrP under both denaturing and non-denaturing conditions are one possible route to achieving this. The binding of the capture antibody to PrP may be affected by it being bound to a solid-phase which may adversely affect the affinity of the antibody for the antigen due to orientation or steric hindrance effects. It is essential that both capture and detection antibodies used in this kind of two-site assay are able to bind simultaneously to the antigen. Optimum binding is dependent upon the antibody epitopes and the relative affinity of the antibody antigen interactions. To assess the effects of different antibodies on assay sensitivity three different monoclonal antibodies were used as capture antibodies with europium labelled 3F4 as detection antibody in CDI assays for the detection of 10 % vCJD brain homogenate spiked into platelet poor plasma. The different capture antibodies used were FH11, D18, and 1120-64-9. Details of epitope specificity, host and immunogen can be found in Table 6. 10% vCJD brain homogenate supplied by NIBSC was spiked into platelet poor plasma in log dilutions between $10^{-0.5}$ – $10^{-5.0}$ in 0.5 log increments ensuring that there was at least 2 mL of each dilution. Each dilution was then aliquoted in 125 µL volumes and stored at -

80° C. Samples from this same dilution series were used in all assays so that sensitivity measurements between assays using different antibodies were directly comparable.

5.2.1 MAbs FH11 & 3F4

Coating conditions for monoclonal antibody FH11 were assessed as described in an earlier titration in section 3.1.1.3 and a concentration of 5 μG/mL was sufficient to saturate microtitre wells with antibody ensuring maximal binding. The vCJD spiked plasma dilution series were assayed twice in duplicate with five plasma samples from healthy adult donors as controls in each assay. Two separate assays were carried out on different days following the protocols for CDI described in the materials and methods section 2.4. The mean cpm for duplicates of native and denatured samples for each spiked dilution were calculated and the ratio of denatured divided by the native counts for each sample were then calculated. The mean ratios and two times the standard deviation were calculated for the two runs of the dilution series and for the five control plasma samples in each assay, the latter providing the cut-off value for the presence of disease associated PrP^{Sc}. A line graph illustrating the data from the first assay is shown in Figure 38.

Figure 38

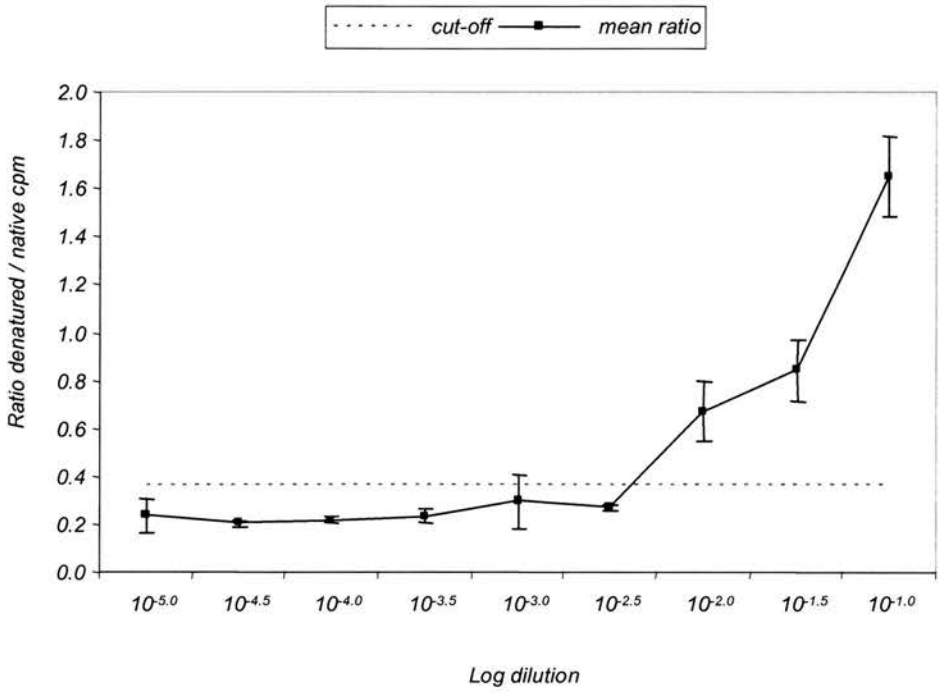


Detection of 10 % vCJD brain homogenate spiked into platelet poor plasma assayed by CDI. Error bars represent ± 2 sd. The cut-off is the mean ratio ± 3 sd calculated from five plasma samples from healthy adults.

PrP^{Sc} in spiked plasma samples can be detected to two log dilutions of a 10 % brain homogenate spiked into platelet poor plasma using CDI employing FH11 as capture antibody and europium labelled 3F4 as detection antibody. The limit of detection has improved by 0.5 logs from the first CDI assay the results of which were shown at he end of the previous chapter in section 4.9, this is most likely due to improved technical operator skills and the use of a fresh aliquot of NIBSC vCJD brain homogenate. As in the previous CDI assay using the same antibodies the ratio of cpm for denatured samples divided by the cpm for native samples are less than one

for the majority of spiked samples and for the unspiked plasma control samples. The level of detection of PrP in denatured samples is therefore less than that detected in the same samples under native conditions. This is most likely due to a reduced affinity of MAb FH11 for denatured PrP. The use of MAb 3F4 in PrP western blot analysis and differential extraction assays demonstrate that this antibody has a high affinity for denatured PrP and so it is unlikely to be the cause. If antibodies detected both native and denatured PrP with the same affinity then you would expect ratios in unspiked plasma samples to be close to one. The same findings are also characteristic of the second CDI assay using this antibody pairing. The results are shown in Figure 39 below.

Figure 39



Detection of 10 % vCJD brain homogenate spiked into platelet poor plasma assayed by CDI. Error bars represent ± 2 sd. The cut-off is the mean ratio ± 3 sd calculated from five plasma samples from healthy adults.

A limit of detection of 2 log dilutions of 10 % vCJD brain homogenate is the same as in the previous assay and has the same sensitivity as the differential extraction method for the detection of PrP^{Sc} in spiked plasma samples described in section 4.7 which uses the same pair of monoclonal antibodies for capture and detection. Denaturation of the antibody reactant can affect the affinity of interaction with monoclonal antibodies; it appears that the affinity of FH11 for PrP is affected in this manner.

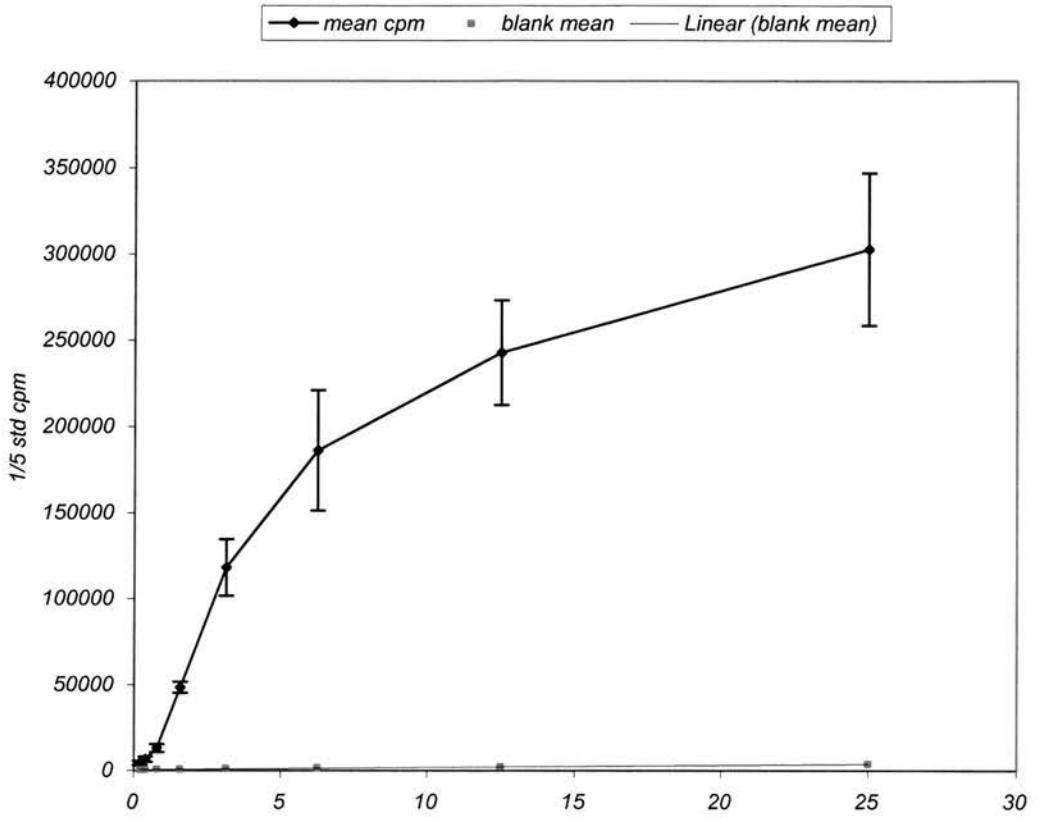
5.2.1 D18 & 3F4

Monoclonal antibody D18 was kindly supplied by Dr Anthony Williamson at the Scripps Institute, La Jolla, California, USA and has been described and characterised previously [24].

5.2.1.1 Titration of coating antibody

D18 antibody has not been used before in two-site DELFIA assays as a capture antibody and therefore required titration to establish coating conditions where the antibody is present at saturating concentrations and antigen capture is maximal. D18 was titrated in exactly the same way as MAb FH11 as described in section 3.1.1.3 in a range between 0.195 and 25 μG/mL. The mean europium counts per minute for samples assayed at each concentration of coating antibody along with the two times standard deviation, to equal 95% confidence interval, were calculated and the data plotted. Counts of background binding to coated wells incubated with assay buffer alone were also plotted to give an indication of how background measurements change with the concentration of coating antibody. The results are plotted in the Figure 40 below.

Figure 40



Assessment of coating conditions for monoclonal antibody D18. Detection of a 1/5 dilution of platelet standard in counts per minute is shown on the y-axis. With increasing concentrations of coating antibody (μG/mL) represented by the x-axis. The error bars represent the mean ± 3 sd. The cpm for blank wells at each antibody concentration are also shown. .

The titration graph shows that capture of PrP^c by D18 increases as the concentration of coating antibody increases but that the titration curve does not flatten off completely in the concentration range tested. Binding has not reached a maximum and the antibody is not present at saturating concentrations. The level of background binding increases as the concentration of coating antibody increases. Comparing this titration with that of FH11, FH11 gives much higher binding of PrP^c than D18 at lower antibody concentrations and it reaches saturation at lower concentrations.

These differences illustrate how epitope affinity and avidity differences between antibodies affect binding in this assay.

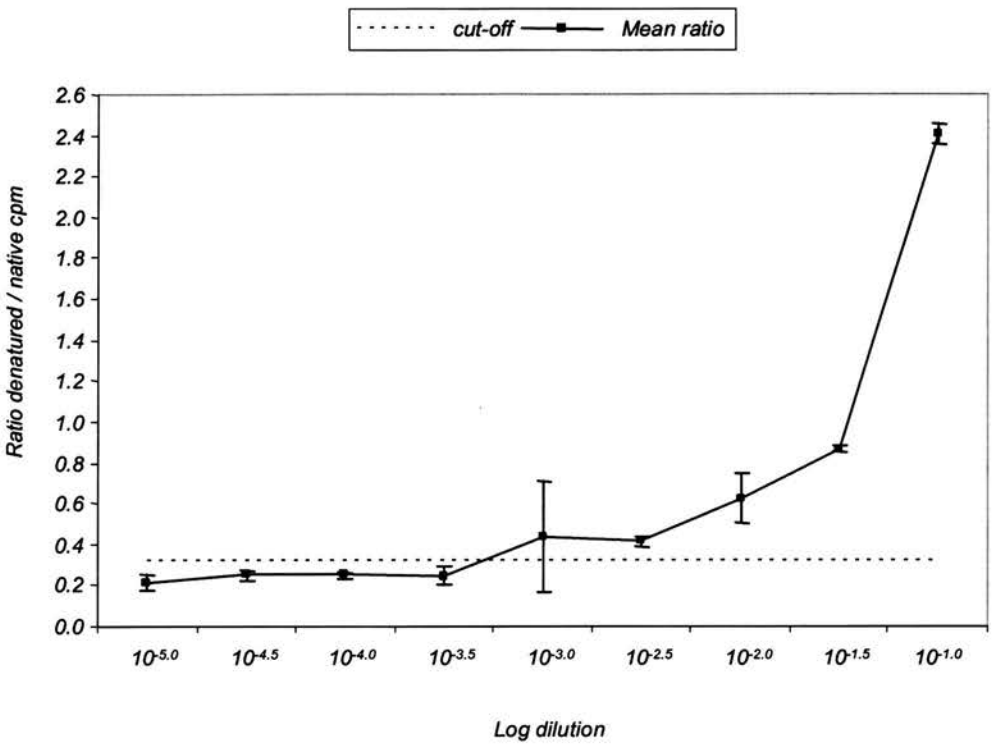
The titration results for D18 present certain difficulties in its use as a capture antibody. Ideally it is advisable to use antibody at concentrations where binding has reached a maximum, where any further increases in antibody concentration do not further enhance binding. In the case of D18, binding is still increasing at a concentration of 25 μG/mL. Despite this it would not be economical to use D18 at this or higher concentrations since you would require 525 μG of antibody at this concentration to coat one microtitre plate. An additional factor to consider is that background binding is at high levels where coating concentrations are high. The use of D18 as a capture antibody requires a compromise between achieving the highest level of antigen capture without excessive levels of background binding given that it would require large quantities of antibody to achieve saturation and that the use of high concentrations of antibody would be associated with high background binding and would be expensive. A coating concentration of 10 μG/mL would ensure economical use of antibody resources, comparatively low levels of background binding less than 2000 cpm, and relatively high degree of antigen capture. It is worth pointing out that the level of antigen capture using D18 at this concentration is approximately half that you would expect using FH11 at 5 μG/mL.

5.2.1.2 CDI assay of spiked plasma samples

Conformation dependent immunoassay of the spiked plasma series was carried out exactly as with the FH11 and europium labelled 3F4 antibody pairing described in 5.2. The results of the mean ratio of cpm for denatured samples divided by those for

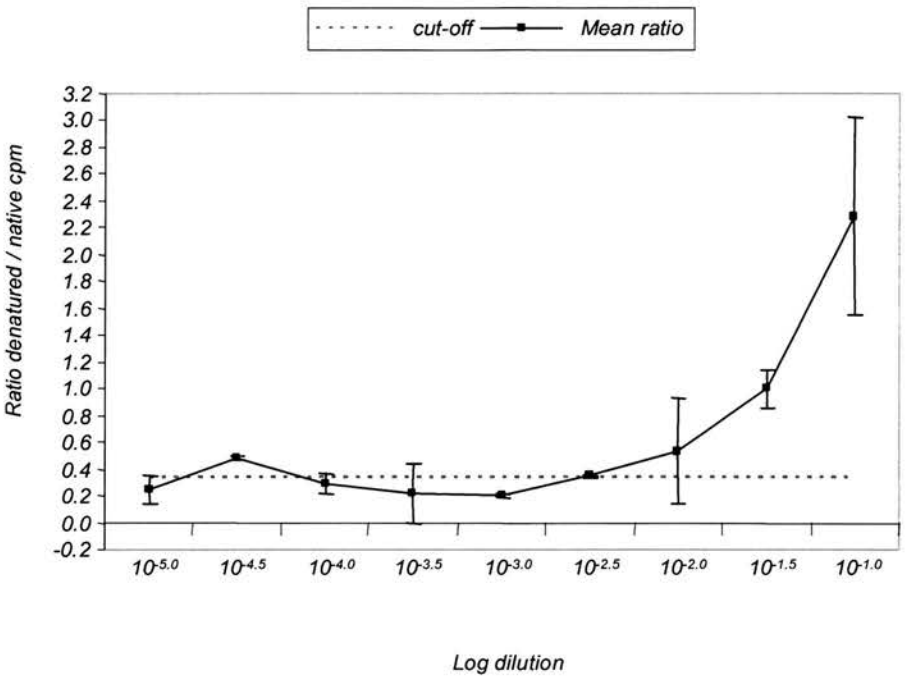
the native sample for both runs of the spiked dilution series assayed twice in separate assays are shown in Figures 41 and 42 below.

Figure 41



Detection of 10 % vCJD brain homogenate spiked into platelet poor plasma assayed by CDI. Error bars represent ± 2 sd. The cut-off is the mean ratio ± 3 sd calculated from five plasma samples from healthy adults.

Figure 42



Detection of 10 % vCJD brain homogenate spiked into platelet poor plasma assayed by CDI. Error bars represent ± 2 sd. The cut-off is the mean ratio ± 3 sd calculated from five plasma samples from healthy adults.

Both assays produce similar results with a limit of detection above cut-off for 10 % vCJD brain homogenate of between $10^{-2.5}$-$10^{-3.0}$ logs. In a comparison with capture antibody FH11, D18 exhibits a superior sensitivity and higher ratios for the more dilute spiked samples. However D18 also shows ratios of < 1 for the plasma control and higher log dilutions of spiked samples as was seen with FH11, and indicates a change in antibody affinity as a consequence of denaturation with GdnHCL.

5.2.2 1120-64-9 & 3F4

Monoclonal antibody 1120-64-9 was kindly supplied by Dr Martin Vey and Dr Albrecht Gröner from Aventis Behring GmbH, Marburg, Germany. The production of this anti-prion monoclonal antibody and its use in conformation-dependent immunoassay have been described previously [152]. Epitope, reactivity and immunogen information for this antibody are shown in table 6 (page 82).

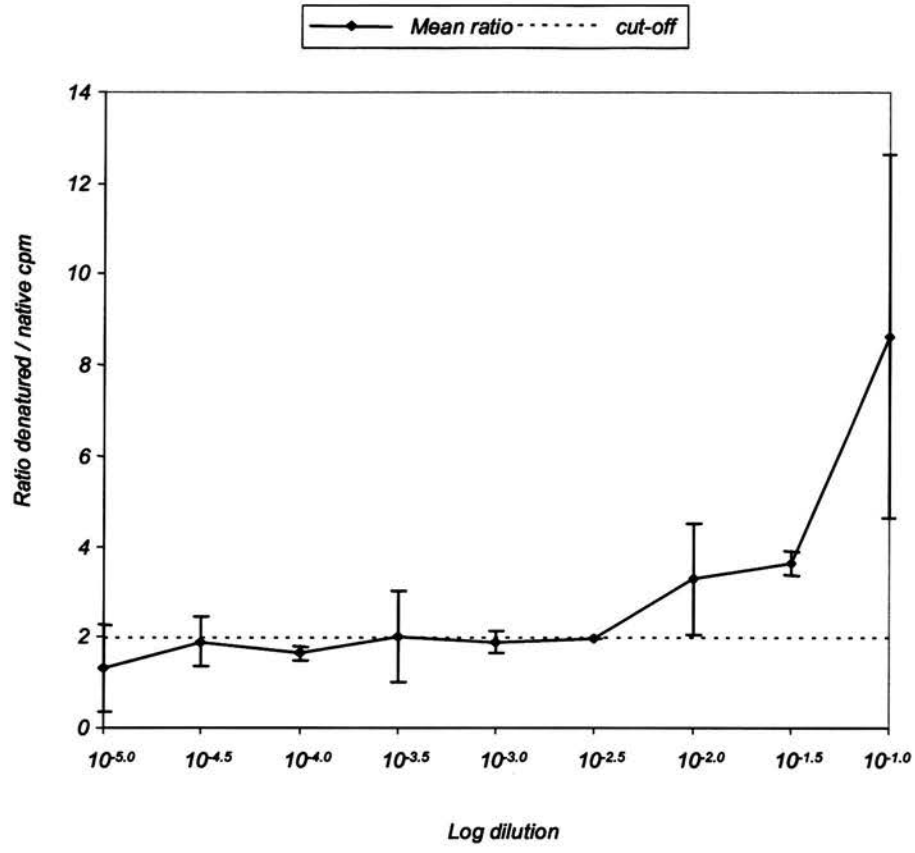
5.2.2.1 Titration of coating antibody

We had a limited quantity of this antibody and its use in conformation-dependent immunoassays had previously described, so rather than use antibody performing our own titration experiments microtitre plates were coated with 10 μG/mL of antibody as recommended [152]. A personal communication from Dr Albrecht Gröner confirmed that the antibody is present at saturation at this concentration and maximal binding is reached.

5.2.2.2 CDI assay of spiked plasma samples

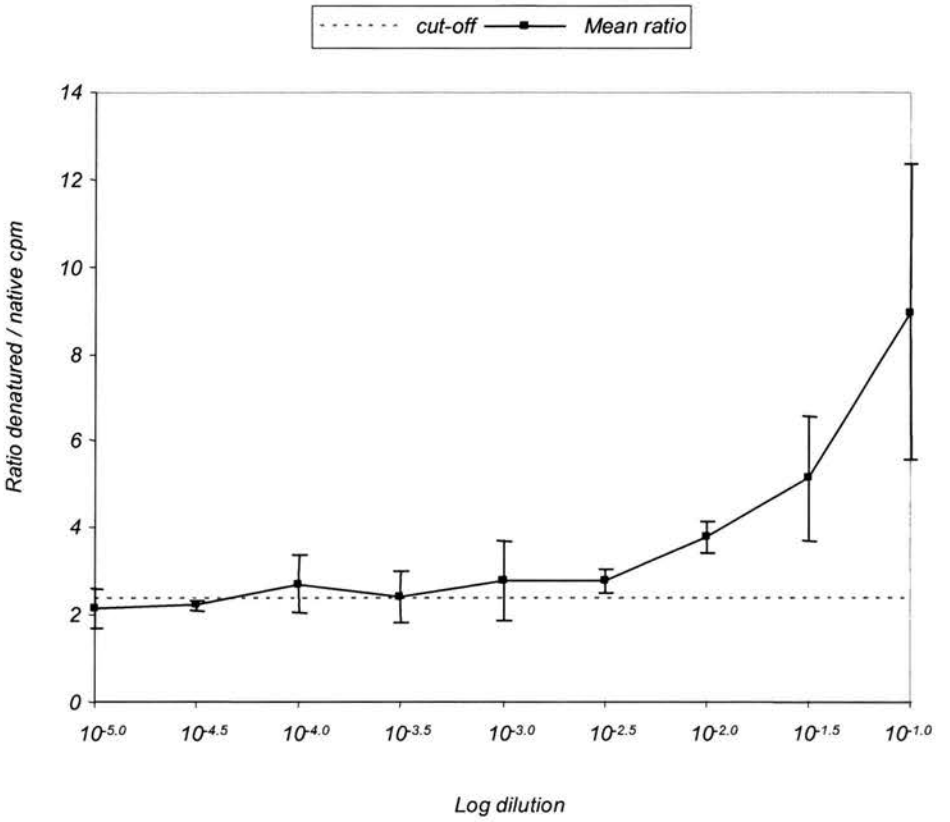
Conformation-dependent immunoassay was carried out exactly as described above to ensure valid comparisons between different antibody pairings. Figures 43 and 44 illustrate the results from the two assays.

Figure 43



Detection of 10 % vCJD brain homogenate spiked into platelet poor plasma assayed by CDI. Error bars represent ± 2 sd. The cut-off is the mean ratio ± 3 sd calculated from five plasma samples from healthy adults.

Figure 44



Detection of 10 % vCJD brain homogenate spiked into platelet poor plasma assayed by CDI. Error bars represent ± 2 sd. The cut-off is the mean ratio ± 3 sd calculated from five plasma samples from healthy adults.

The conformation-dependent immunoassays performed using this antibody do not show greatly increased sensitivity for the detection of PrP^{Sc} compared with using FH11 or D18 as capture antibodies. The first assay has a limit of detection of two logs dilution above cut-off, and in the second assay the ratio of counts flattens off and does not decrease with dilution as the assay reaches two and a half log dilutions. With a sensitivity of between two and two and a half log dilutions the assay performs as well as FH11 but not as well as D18 which has a higher sensitivity. What is

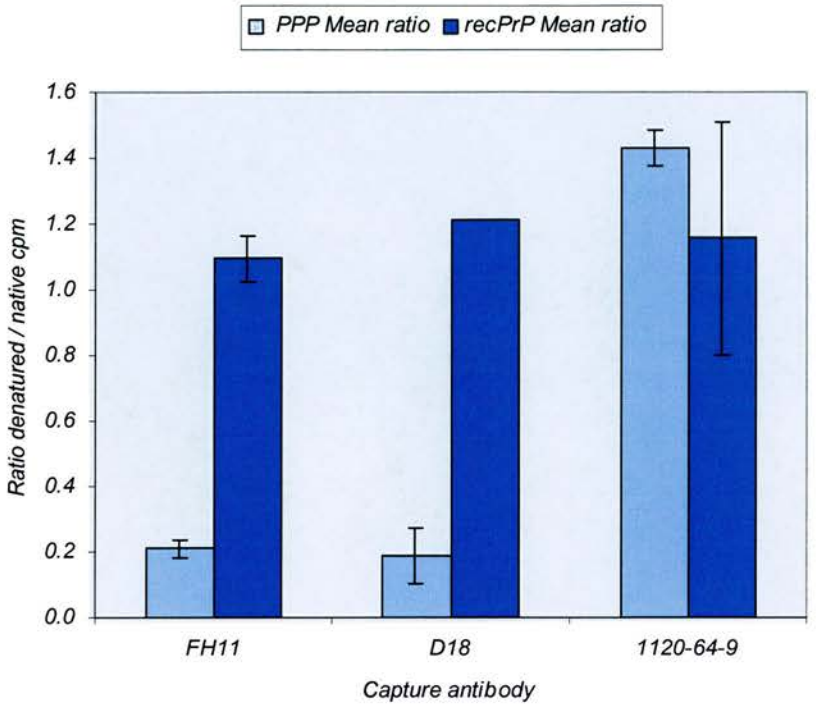
striking about the results using monoclonal antibody 1120-64-9 is that the ratios for all spiked and control samples are greater than one. This is significant since this is the only capture antibody which does not exhibit different patterns of binding to native and denatured samples. The mean ratio from 5 control plasma samples assayed is 1.683. This is higher than 1.0 due to exposure of the 3F4 epitope associated with denaturation of protein to a monomeric form. Although this epitope is fully exposed in PrP^{c}, denaturation will allow slightly better interaction of the detection antibody with its epitope thus explaining a ratio between 1.0 and 2.0. Given the ratio of the control samples it can be stated that the capture antibody is capturing just as much PrP in denatured samples as is captured in native samples. The 3F4 epitope is hidden in PrP^{Sc} but accessible after denaturation with GdnHCL. Therefore in the log dilutions where the ratio is higher than the cut-off, PrP^{Sc} contributed by the spike of vCJD brain homogenate can be detected, and the increased ratio is a direct consequence of increased binding to the 3F4 epitope after denaturation with GdnHCL. The values of ratios for spiked plasma samples are much more separated with monoclonal antibody 1120-64-9 between approximately 2.0 and 9.0 for spiked dilutions 10^{-1} $-10^{-2.5}$ with good separation between dilutions in contrast with using capture antibodies FH11 and D18 where ratios for this same series of dilutions are concentrated within less than a range of two ratios. Good separation between dilutions is essential to ensure the clear definition of positive samples above cut-off.

5.3 *A study of the interaction of different capture antibodies with PrP^c*

To study the interaction of these three different capture antibodies with PrP^c more closely we set up an experiment comparing the detection of PrP^c in platelet poor plasma from healthy adult donors and in recombinant human PrP^c obtained from Prionics, Zurich, Switzerland.

Two rows of a microtitre plate were coated for each of the three monoclonal capture antibodies FH11, D18, 1120-64-9, using concentrations determined in the previous experiments. Six aliquots of 100 µL platelet poor plasma and six aliquots of 100 µL recombinant PrP^c at a concentration of 50 µG/mL were assayed following the CDI protocol described in materials and methods section 2.4. For each of the three different capture antibodies two samples each of platelet poor plasma and recombinant human PrP^c were assayed under native and denatured conditions in triplicate. The mean ratios and two times the standard deviation were calculated and the results illustrated in Figure 45. Europium labelled monoclonal antibody 3F4 was used as the detection antibody in this assay.

Figure 45



Ratio of platelet poor plasma (PPP) and recombinant PrP^c assayed by CDI using three different capture antibodies. Error bars show the mean ratio ±2 sd.

This assay permits the assessment of the capture of both PrP^c present in platelet poor plasma and recombinant PrP^c by three different antibodies. Because the epitope of the detection antibody 3F4 is more fully exposed in PrP^c, differences in the CDI ratio should reflect the relative affinity of each capture antibody under native and denaturing conditions and the suitability of antibody pairs. The CDI ratios for the human recombinant PrP^c are all very similar despite the use of different capture antibodies with ratios in a range between 1.095 – 1.21. However the ratios for the detection of PrP^c in platelet poor plasma are extremely low for MAbs FH11 and D18 at a ratio of approximately 0.2 but much higher with MAb 1120-64-9 at 1.43. These figures support results found for each different antibody pair with platelet poor plasma samples assayed to ascertain cut-off values in CDI analysis of spiked samples

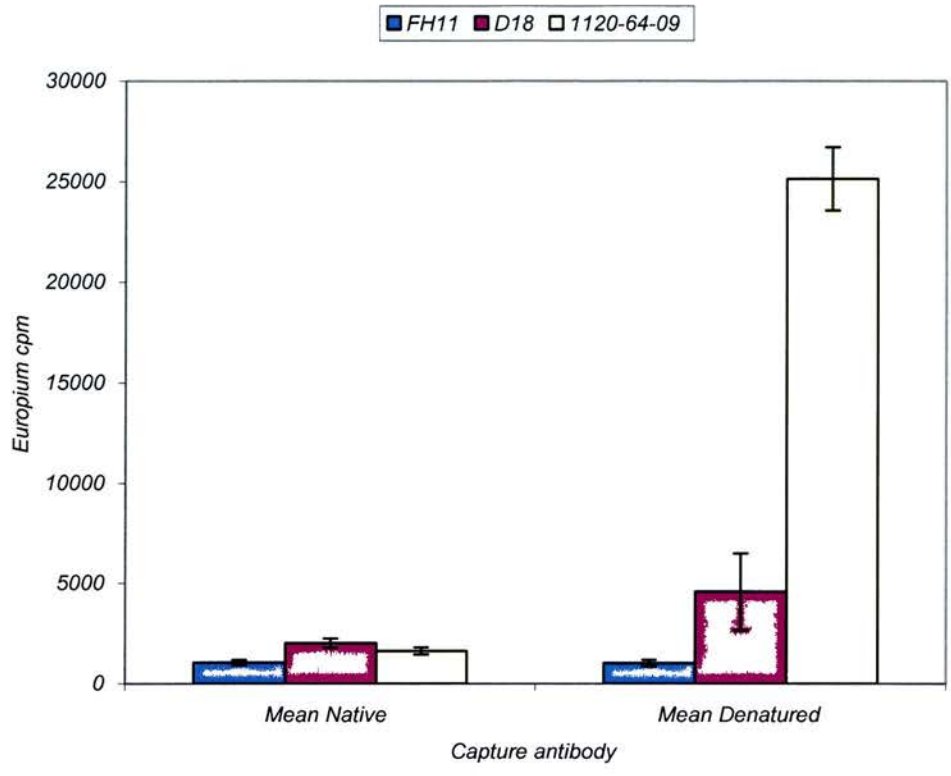
in section 5.2. The results indicate that monoclonal antibodies FH11 and D18 capture less PrP^c after denaturation compared to assaying the native sample hence the ratio of less than one. An interesting finding is that ratios are similar for all antibodies when purified recombinant human PrP^c is assayed. This would appear to rule out the possibility of the disruption of discontinuous epitopes after denaturation as a cause for the reduced ratios with MAbs FH11 and D18. It may be that there are proteins present in platelet poor plasma which bind non-specifically with MAb FH11 and MAb D18, and compete with PrP^c for binding to the antibody. The antibodies affinity for denatured treated PrP^c may be altered therefore allowing interaction with other proteins. It may also be that MAbs FH11 and D18 are more specific for the conformation of native PrP^c and that the affinity is reduced in denaturing conditions. It is significant also to note that the immunogen for 1120-64-9 was human PrP^c whereas MAb FH11 was raised using recombinant ovine PrP^c and MAb D18 with purified PrP27-30 from experimental scrapie in hamsters. The findings here may simply reflect the fact that MAb 1120-64-9 has a much higher affinity for human PrP^c which is retained even after denaturation. With MAbs FH11 and D18 the relative affinity for human PrP^c is reduced after denaturation and the interaction between paratopes and epitope maybe subject to interference from other cross-reactive factors in human plasma.

5.4 *A study of the interaction of different capture antibodies with PrP^{Sc}*

To assess the suitability of the same three antibody pairs for the detection of PrP^{Sc} an experiment similar to that described above was carried out. One row of a microtitre plate was coated with each of the three different capture monoclonal antibodies (FH11, D18, 1120-64-09) using coating conditions as described in 5.3. Six 100 µL aliquots of 10 % vCJD brain homogenate supplied by NIBSC were treated with 50 µG/mL PK for one hour at 37 ° C and Pefabloc added to a final concentration of 1 mM. Two samples were assayed by CDI in triplicate for each of the three different coating antibodies following methods described in section 2.4.

Because the 3F4 epitope is occluded in PrP^{Sc} the interaction of 3F4 under native conditions will be low and therefore you would expect a low level of binding for all of the different antibody pairs. Under denaturing conditions PrP^{Sc} will unfold exposing the 3F4 epitope and permitting the binding of the antibody. The level of europium counts obtained for each antibody pair under denaturing conditions will indicate the suitability of each antibody pair for the capture and detection of PrP^{Sc}. Figure 46 below illustrates the europium counts obtained for PrP^{Sc} samples assayed under native and denaturing conditions for each of the three antibody pairs.

Figure 46



Europium cpm for the detection of PrP^{Sc} under native and denatured conditions captured with either monoclonal antibody FH11, D18, or 1120-64-09. Europium labelled 3F4 was employed as detection antibody, error bars represent ± 2 sd.

As predicted the europium signals for PK treated vCJD brain homogenate under native conditions are low for all three antibody pairs. This is a consequence of the epitope for the detection antibody remaining hidden in PrP^{Sc}. The residual signal probably reflects the small amounts of PrP^{c} remaining in the brain homogenate which were not degraded with PK treatment. The low signal may also represent minor changes in conformation of PrP^{Sc} as a result of its binding to the capture antibody, which permit a low affinity interaction between 3F4 and its epitope.

Using MAbs D18 and 1120-64-09 the detection of PrP by MAb 3F4 was increased following denaturation. The europium signal obtained with FH11 and 3F4 does not show any increase. Europium counts are lowest with capture antibody FH11 due to the fact that very little PK treated PrP^{Sc} is captured by this antibody. Reference to Figure 2 shows that FH11 has two epitopes in the N-terminus of PrP. Also shown on this diagram are the two major sites of PK cleavage, and these illustrate that the first FH11 epitope which spans amino acid (aa) residues 46-58 is N-terminal to both sites of PK cleavage. In addition one of the cleavage sites lies within the second FH11 epitope which spans amino acid residues 90-101. Therefore after PK treatment of vCJD brain homogenate, cleavage of PrP will seriously disrupt the ability for FH11 to interact with its epitopes. It is likely that MAb FH11 will capture N-terminal fragments which have been cleaved from the rest of PrP and do not have the 3F4 epitope. This would explain the lack of increase in europium counts when the denatured samples are assayed.

The D18-3F4 pairing shows a 2.26 fold increase in signal with denaturation. By far the best pairing is 1120-64-09 - 3F4 which shows a 15.4 fold increase in signal. These differences in signal increases may reflect the capture of PrP^{Sc} at different epitopes which may govern the success of the interaction of MAb 3F4 with its epitope. Monoclonal antibody 1120-64-09 captures PrP in the C-terminal region somewhere between amino acid residues 175 –230, and D18 captures PrP between residues 133-157. The 3F4 epitope is aa 109-112 and it is plausible that a capture epitope distal to the epitope of the detection antibody such as that of 1120-64-09 may permit better interaction of the detection antibody than a capture epitope which is

closer for example that of D18. It may simply be a case of steric hindrance where capture of the antigen in a particular manner permits better physical interaction of the detection antibody.

The experimental studies presented in this chapter investigated the suitability of three monoclonal antibodies for the capture of PrP in a CDI immunoassay format. Affinity differences for the three antibody pairs for binding to PrP^{c} and PrP^{Sc} produce differences in CDI ratios for spiked plasma samples.

Chapter 6: Studies into the purification and concentration of PrP^{Sc}

6.1 Background

In order to improve the sensitivity of the CDI DELFIA assay system for the detection of PrP^{Sc} and to permit the analysis of larger volumes of sample, sodium phosphotungstate (NaPTA) was used to precipitate PrP^{Sc}. This methodology has been described and used previously in these types of assays by Safar et al [21] and others [152] to precipitate PrP^{Sc}. Safar et al claim that NaPTA complexes with oligomers and polymers of PrP^{Sc} but not PrP^{c} in the presence of magnesium chloride and neutral pH. These complexes are collected by high-speed centrifugation. Here these methods have been adapted and are described in the materials and methods section 2.4.1. The use of this precipitation method permits the isolation of PrP^{Sc} from larger volumes of spiked samples and is suited to tissues in which PrP^{Sc} may be present in low concentrations.

6.2 Detection of PrP^{Sc} in spiked plasma samples using NaPTA precipitation.

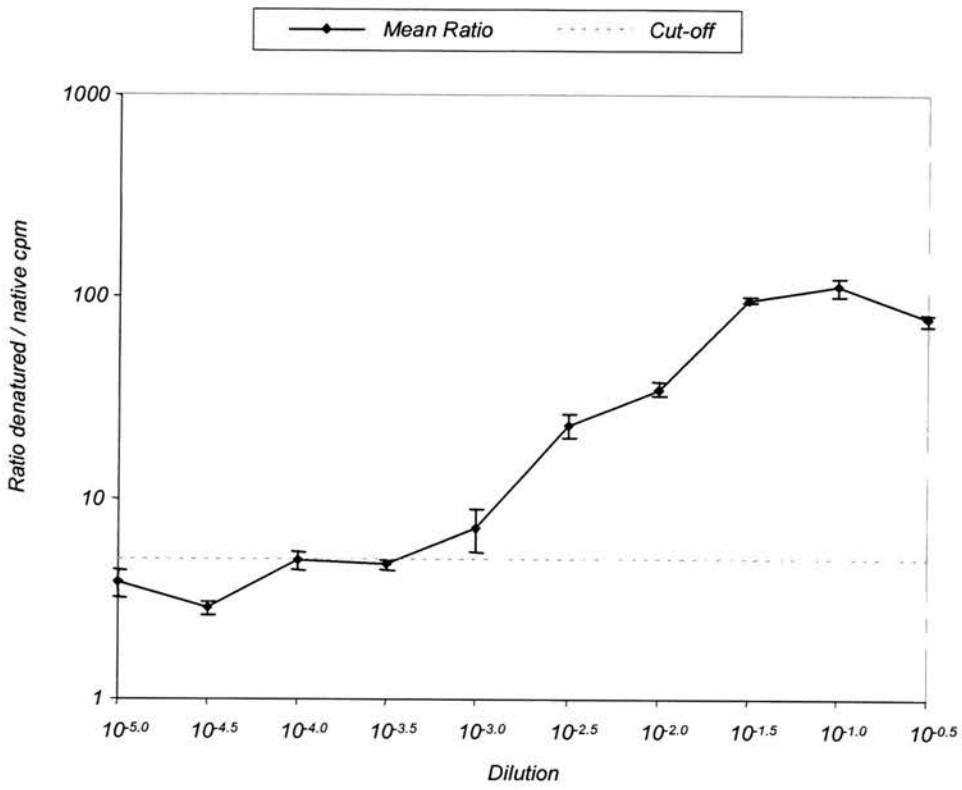
A dilution series of platelet poor plasma samples spiked with 10 % vCJD brain homogenate provided as a standard by NIBSC was prepared in 0.5 log increments between 0.5 to 5.0 log dilutions. 900 µL of each spiked sample was treated with NaPTA and analysed by CDI as described in section 2.4. It is important to note that in these experiments 900 µL of each spiked dilution is subject to NaPTA precipitation and the 50 µL resuspended volume is analysed by CDI. You would therefore expect the sensitivity for the detection of PrP^{Sc} to be in the region of 0.9 logs greater than in the previous CDI experiments where 100 µL of spiked sample

was assayed assuming that the recovery of PrP^{Sc} by this method is unaffected by increases in sample volume. The first 0.9 log increase may simply be a consequence of analysing a nine-fold greater sample volume.

Spiked and control plasma samples were assayed in triplicate on a microtitre plate precoated with 10 μG/mL of monoclonal antibody 1120-64-09. Captured PrP was detected using europium labelled monoclonal antibody 3F4.

The mean ratios of denatured europium counts divided by the native counts were plotted for each spiked sample and the limit of detection assessed against a cut-off provided by unspiked human control plasma samples plus three times the standard deviation. The results for this spiking experiment are plotted in Figure 47.

Figure 47



NaPTA concentration and CDI analysis of vCJD brain homogenate spiked into human plasma. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio of unspiked control human plasma ± 3 sd.

The limit of detection is 10^{-3} against a cut-off of control plasma samples. Comparison with a previous experiment where 100 µL of the same spiked plasma sample series were assayed with the same antibody pairing (section 5.2.3) the limit of detection in this previous experiment was 10^{-2}. This finding was expected considering that PrP^{Sc} was concentrated from a sample nine times greater in volume than that assayed in 5.2.3. These results illustrate that NaPTA can be used as a means of precipitating PrP^{Sc} from larger sample volumes. There are considerable

increases in the CDI ratios obtained with spiked samples after NaPTA precipitation compared with the spiked samples assayed in 5.2.3 see Table 22.

It is claimed that NaPTA binds and precipitates PrP^{Sc} but not PrP^{c}. Therefore would not expect PrP^{c} to be present in large quantities in NaPTA precipitated samples. In the absence of large quantities of PrP^{c} there will be less competition between PrP^{c} and PrP^{Sc} for binding to the capture antibody. NaPTA precipitated samples should give higher ratios since it is claimed that NaPTA binds to PrP^{Sc} but not PrP^{c}. This is indeed the case with sample ratios highly elevated in this experiment compared with smaller volumes of the same spiked plasma sample series assayed in experiments described in section 5.2.3. Differences in ratios for each spiked sample dilution are shown in the Table 22.

Table 22

Log dilution	Mean Ratio + NaPTA	Mean Ratio - NaPTA	Fold increase
$10^{-5.0}$	3.820	1.313	2.909
$10^{-4.5}$	2.821	1.894	1.489
$10^{-4.0}$	4.873	1.648	2.958
$10^{-3.5}$	4.647	2.017	2.304
$10^{-3.0}$	7.138	1.890	3.777
$10^{-2.5}$	23.249	1.968	11.814
$10^{-2.0}$	34.856	3.293	10.584
$10^{-1.5}$	96.942	3.629	26.711
$10^{-1.0}$	112.188	8.632	12.996

Ratios of spiked plasma samples assayed by CDI with and without NaPTA pre-treatment.

The data shown in Table 22 illustrates the considerable increases in CDI ratio obtained with NaPTA precipitation. As mentioned above at least a nine-fold increase in sensitivity is expected due to a nine-fold difference in the volume of sample assayed in these two separate experiments. What is unclear though is whether

NaPTA is in fact specifically binding PrP^{Sc}. Reference to the CDI ratio of 4.27 generated by NaPTA precipitation of control unspiked plasma samples from healthy adults would suggest that NaPTA also precipitates PrP^{c} and possibly other protein factors present which bind non-specifically to capture and detection antibodies. PrP^{c} shows a relatively small increase in antibody binding with denaturation so it would appear that additional factors are interacting with the capture antibody.

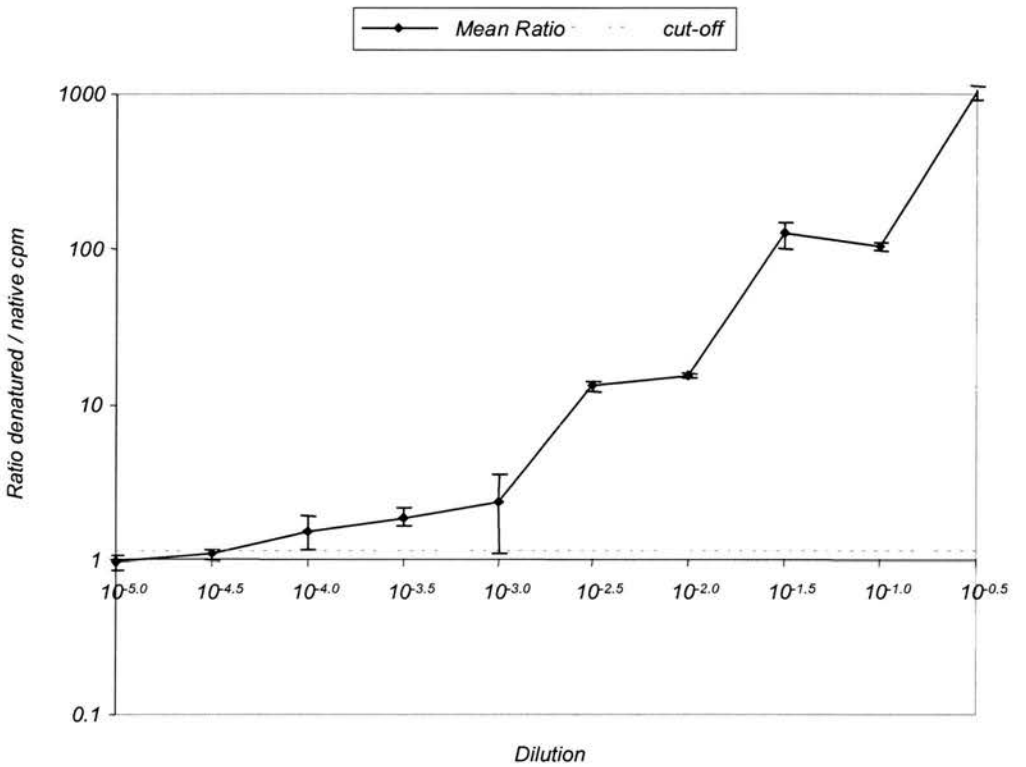
It is likely that NaPTA effectively precipitates not only PrP^{Sc} but also additional protein factors from samples including PrP^{c}, and that it is the specificity of the capture antibody 1120-64-09 which mediates the binding of PrP. If NaPTA precipitation was specific for PrP^{Sc} alone then the use of PK should not increase the assay sensitivity unless N-terminal cleavage of PrP^{Sc} improves its interaction with the capture antibody. The epitope for this antibody is situated in the carboxyl terminus and you would not expect N-terminal cleavage to improve binding. If NaPTA precipitated PrP^{Sc} alone then it is more likely that you would expect PK to degrade some of the PrP^{Sc} reducing the level present and so in-turn decreasing the sensitivity of its detection. The use of PK in these assays is considered in the section below.

6.3 Use of PK as a pre-treatment to NaPTA precipitation and CDI analysis.

To consider whether the pre-treatment with PK is sufficient to increase the sensitivity of the CDI assay for the detection of PrP^{Sc}, log dilutions of vCJD 10 % brain homogenate spiked into platelet poor plasma (as prepared in section 6.2) were pre-treated with 250 μG/mL PK for one hour at 37 ° C. The proteolysis was stopped

with the addition of Pefabloc to a final concentration of 1 mM. This experiment was carried out exactly as that performed in section 6.2 except that each log dilution was pre-treated with PK. The ratio of the denatured sample divided by the native sample europium counts are plotted against log dilution in the Figure 48 below.

Figure 48



NaPTA concentration and CDI analysis of vCJD brain homogenate spiked into human plasma. Spiked plasma samples are pre-treated with 250 µG/mL PK. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio of unspiked control human plasma ± 3 sd.

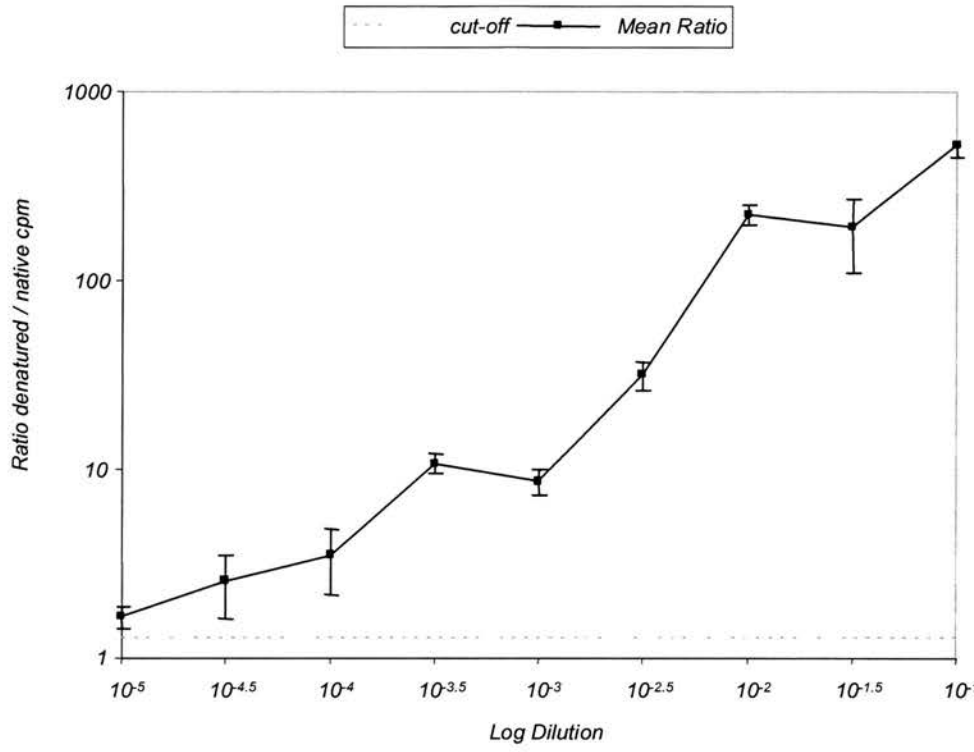
In comparison with the experiment carried out with the same vCJD spiked plasma series in section 6.2 the effect of pre-treatment with PK is to increase the sensitivity for the detection of PrP^{Sc}. This is increased from three logs in the absence of PK treatment to four logs dilution of 10 % vCJD brain homogenate spiked into platelet

poor plasma. PK degrades PrP^c and other proteins which may be precipitated by NaPTA, and it is likely that its removal reduces competition between PrP^c and PrP^{Sc} for binding to the capture antibody ensuring that PrP^{Sc} is bound and subsequently detected after denaturation by 3F4. This explains the increase in sensitivity for the detection of PrP^{Sc}. The cut-off value (the ratio for unspiked platelet poor plasma plus three times the standard deviation) is reduced considerably after PK treatment compared to the cut-off value obtained in the experiment described in section 6.2 which is also a likely consequence of the degradation of PrP^c in plasma samples. These results suggest that the NaPTA precipitation is not specific for PrP^{Sc}.

6.3.1 CDI analysis of PK treated vCJD spiked plasma samples using neurological control brain homogenate as cut-off.

The previous experiment described in 6.3 was reproduced but incorporated a non-CJD neurological control using an Alzheimer's disease brain homogenate as control to provide the cut-off value. The results from this experiment are shown in Figure 49 below. They illustrate that PrP^{Sc} can be detected to 5 logs dilution above the cut-off. This is equivalent to a minimum detectable volume of 9 nL of a 10 % vCJD brain homogenate. This is an extremely sensitive measure for PrP^{Sc} and is very similar in sensitivity to the findings of Bellon et al [152].

Figure 49



NaPTA concentration and CDI analysis of vCJD brain homogenate spiked into human plasma. Spiked plasma samples are pre-treated with 250 µG/mL PK. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio ± 3 sd of unspiked 10 % brain homogenate from an Alzheimer's disease case.

The increases in sensitivity seen in this assay compared with the previous assay are likely to be a consequence of normal variability in the success of NaPTA precipitation. After NaPTA precipitation and centrifugation sample pellets vary in appearance. Sometimes an insoluble white chalky precipitate of magnesium salts forms making the precipitated material difficult to solubilise and resuspend. This may affect interaction of antibodies with PrP^{Sc} thereby reducing its detection by CDI.

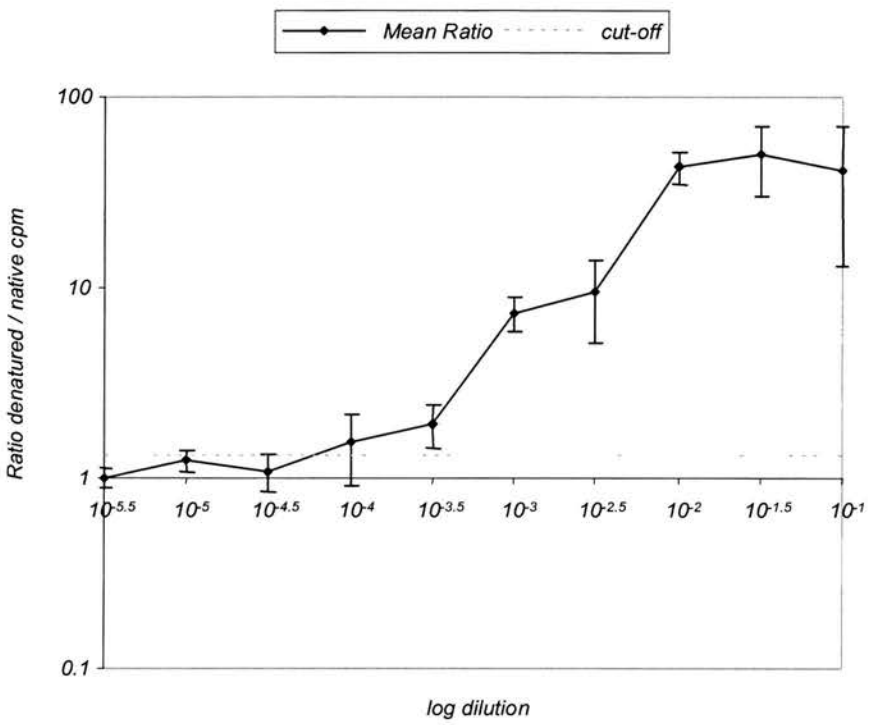
6.4 An investigation into the use of Sodium Chloride as a pre-step precipitation method for the detection of PrP^{Sc} by CDI.

Results generated by experiments performed in 6.2 and 6.3 illustrate that NaPTA does not appear to bind PrP^{Sc} alone. Were NaPTA to bind and precipitate PrP^{Sc} alone then the use of PK should not increase the limit of detection of PrP^{Sc} unless proteolysis of PrP^{Sc} improves interaction with the capture antibody. The treatment of samples with PK prior to NaPTA precipitation increases the sensitivity for the detection of PrP^{Sc}. These results suggest that increases in sensitivity are due to degradation of PrP^{c} and perhaps additional proteinaceous factors which compete with PrP^{Sc} for binding to capture antibody 1120-64-09 in the CDI assay. Given these findings it is possible that the effect of NaPTA is to precipitate all proteinaceous material in a sample much like the traditional use of sodium chloride for these purposes known as 'salting out'. The specificity of the monoclonal antibodies used in the CDI assay ensure that only PrP is captured and the use of PK as a pre-treatment ensured the removal of PrP^{c} and other PK sensitive proteins.

As an alternative to NaPTA precipitation the use of sodium chloride (NaCl) as a precipitation method was assessed using the same vCJD spiked platelet poor plasma log dilution series as used in experiments described in sections 6.2 and 6.3. The use of 10 % NaCl for the precipitation of PrP^{Sc} following treatment with PK has been described previously by Polymenidou et al [160]and is thought to precipitate 97 % of PrP^{Sc} along with other proteins present in a given sample Methods were adapted for the analysis of spiked plasma samples by CDI and are described in section 2.4.2. The CDI ratios for each spiked platelet poor plasma sample are plotted in Figure 50

below. An Alzheimer's disease non-CJD control 10 % brain homogenate was assayed to provide the cut-off.

Figure 50



NaCl precipitation and CDI analysis of vCJD brain homogenate spiked into human plasma. Spiked plasma samples are pre-treated with 250 µG/mL PK. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio ± 3 sd of unspiked 10 % brain homogenate from an Alzheimer's disease case.

This assay shows that 10 % NaCl can be used with some success to precipitate proteins including PrP^{Sc} from PK treated spiked plasma samples for analysis by CDI. This result shows a sensitivity of four log dilutions of 10 % vCJD brain homogenate spiked into platelet poor plasma which is equal to that obtained with the first NaPTA precipitation experiment shown in Figure 47. This is particularly interesting since it

shows that simple protein 'salting out' achieves a similar sensitivity to the use of NaPTA.

Both NaPTA and NaCl can be used for the precipitation and concentration of PrP^{Sc} from large sample volumes prior to analysis by CDI. It does not appear that NaPTA is specific for PrP^{Sc} alone. Such methods may permit the detection of PrP^{Sc} in blood and tissues where it is present at very low concentrations by allowing the precipitation of PrP^{Sc} from large sample volumes.

In this chapter NaPTA precipitation combined with a CDI detection assay have been used to measure PrP^{Sc} in platelet poor plasma samples spiked with 10 % vCJD brain homogenate. The screening of 900 µL of each spiked sample dilution had a maximum detection sensitivity for PrP^{Sc} above cut-off equivalent to a 1/1000 000 dilution of wet brain homogenate. These results are extremely similar to those published by Bellon et al [152] who used these methods in almost identical spiking studies of vCJD brain homogenate into human plasma.

Chapter 7: Detection of PrP^{Sc} in lymphoreticular tissues of patients with vCJD.

7.1 Background

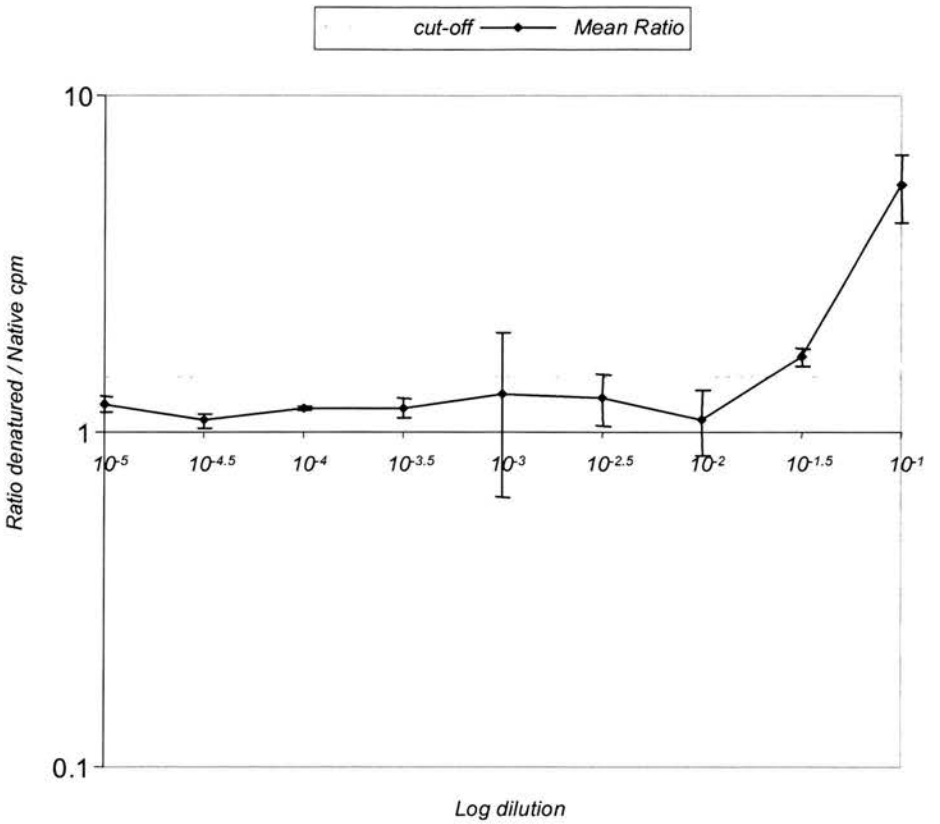
PrP^{Sc} is present at very high concentrations in brain tissue and its use as a source of PrP^{Sc} for the development of diagnostic assays to detect PrP^{Sc} in blood has been criticized over concerns that the PrP^{Sc} found in brain tissue may not be representative of the PrP^{Sc} which you might expect to find in peripheral blood [161]. Its use to assess the sensitivity of assays designed to detect PrP^{Sc} in blood may not be appropriate because PrP^{Sc} if present in blood may differ in its presentation of epitopes, protease sensitivity and glycosylation pattern, all of which may render it unsuitable for detection using methods developed using brain and peripheral tissues as a source of PrP^{Sc}. The blood brain barrier, as its name suggests, does not permit direct interaction of the blood with the brain tissue, but is selective and active in regulating which blood constituents enter the brain, but the blood circulatory system is closely associated with peripheral lymphoreticular tissues such as the spleen, tonsil, and appendix. Immunohistochemistry and western blotting studies have detected the presence of PrP^{Sc} in peripheral tissues of patients with vCJD but not at equivalent levels in patients with sCJD [97]. It is plausible that the PrP^{Sc} present in peripheral tissues is acquired from or exchanged with the blood, which in turn acquires it from the gut associated lymphoid tissue following dietary exposure to BSE. PrP^{Sc} found in peripheral tissues may be closer physiologically to the PrP^{Sc} you might expect to find in the blood of patients with vCJD when compared with brain PrP^{Sc}.

To investigate whether NaPTA precipitation and CDI analysis was suitable for the detection of PrP^{Sc} in peripheral tissues from patients with vCJD, spleen tissue homogenates were assayed.

7.2 NaPTA precipitation and CDI analysis of plasma samples spiked with vCJD spleen tissue.

10 % spleen homogenate prepared in 0.25 M sucrose from a neuropathologically confirmed case of vCJD obtained from NIBSC (Potters Bar, UK) was spiked into platelet poor plasma in a log dilution series prepared in the same manner described for vCJD brain homogenate in section 6.2. 900 µL of each 0.5 log dilution of sample was NaPTA precipitated and analysed by CDI. Unspiked platelet poor plasma samples and 10 % non-CJD spleen homogenate in 0.25 M sucrose (the latter also obtained from NIBSC) were included as assay controls and all samples were assayed without treatment with PK. The CDI ratios for each log dilution are shown in Figure 51 below.

Figure 51



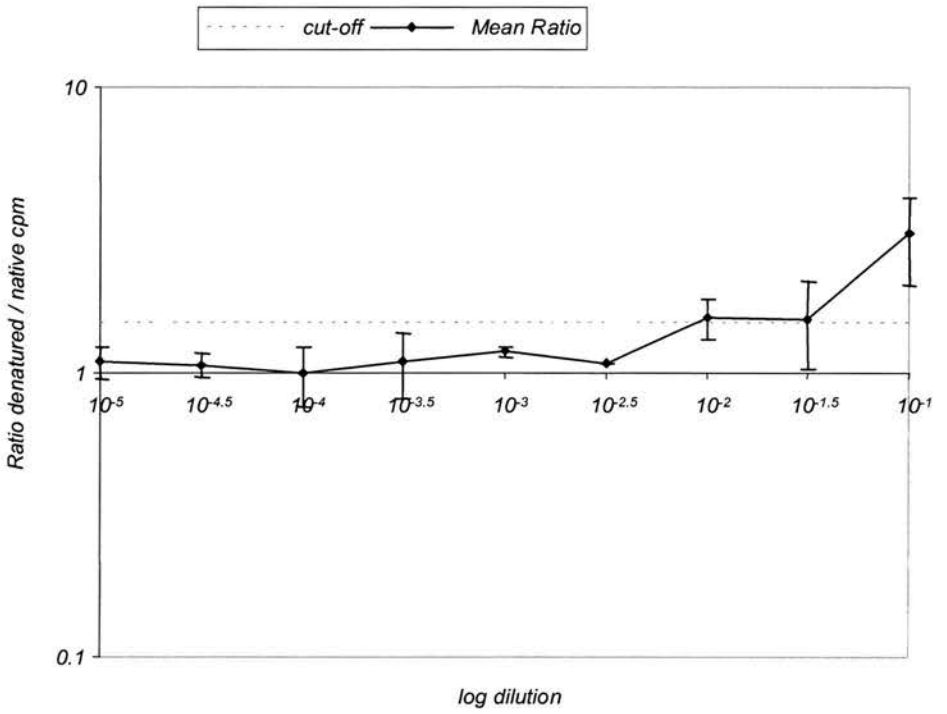
NaPTA concentration and CDI analysis of NaPTA concentrated vCJD spleen homogenate spiked into human platelet poor plasma. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio ± 3 sd of unspiked 10 % spleen homogenate from a non-CJD neurological control.

The presence of PrP^{Sc} in spleen homogenate from a patient with vCJD can be detected using these methods to 1.5 log dilutions of a 10 % homogenate spiked into human platelet poor plasma above a cut-off of non-CJD neurological control spleen homogenate. This sensitivity is half that of the 3 logs found for vCJD 10 % brain homogenate spiked into plasma assayed using these methods. PrP^{Sc} is present at very low levels in spleen homogenate and this is reflected in the low ratios for those positive dilutions, which are a consequence of trying to measure very small amounts of PrP^{Sc} in the presence of very large quantities of PrP^{c}.

7.3 Use of PK as a pre-treatment to NaPTA precipitation and CDI analysis.

Pre-treatment with PK may be sufficient to increase the sensitivity of the CDI assay for the detection of PrP^{Sc} by removing competing PrP^{c}. Log dilutions of vCJD 10% spleen homogenate spiked into platelet poor plasma (as prepared in section 7.2) were pre-treated with 250 μG/mL PK for one hour at 37 ° C. The proteolysis was stopped with the addition of Pefabloc to a final concentration of 1 mM. 900 μL samples were then NaPTA precipitated and assayed by CDI following methods as described in section 7.2. The ratio of denatured sample divided by the native sample europium counts are plotted against log dilution in the Figure 52 below.

Figure 52



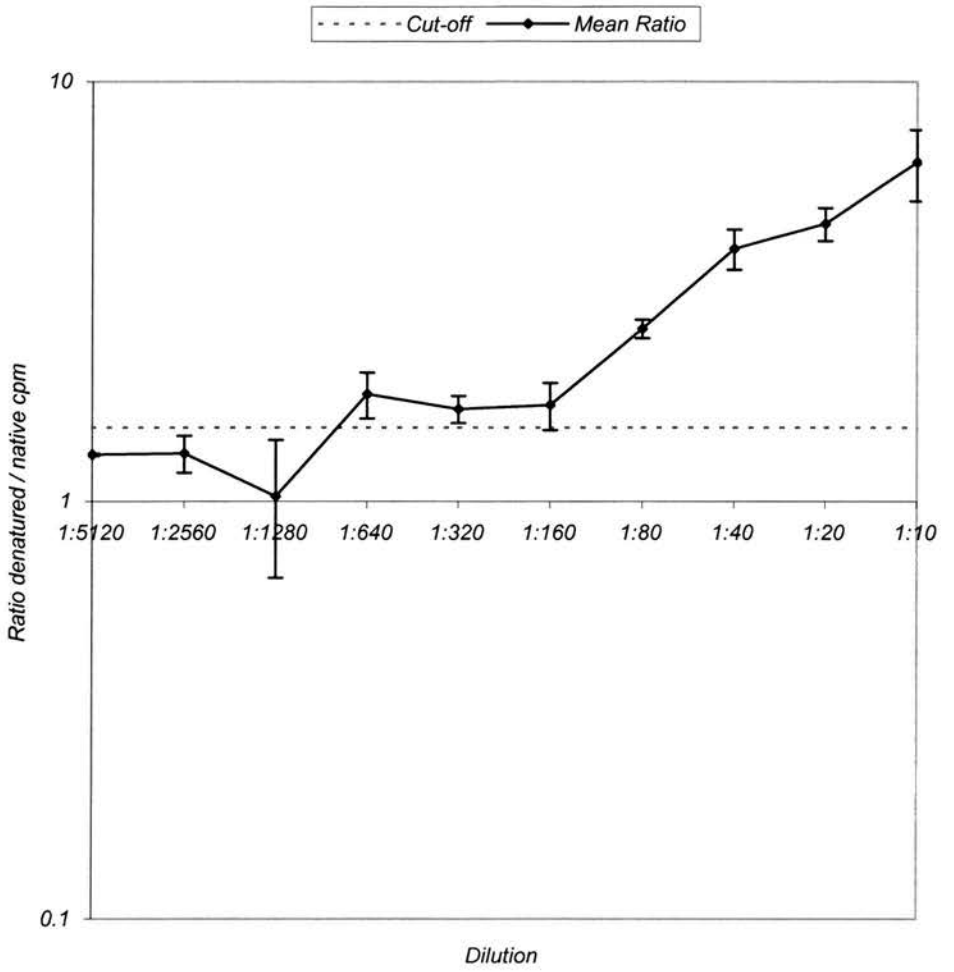
NaPTA concentration and CDI analysis of vCJD spleen homogenate spiked into human plasma. Spiked plasma samples are pre-treated with 250 µG/mL PK. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio ± 3 sd of unspiked 10 % spleen homogenate from a non-CJD neurological control.

Although including PK treatment appears sufficient to increase assay sensitivity, the CDI ratio for the first log dilution is less at 3.069 than the 5.045 obtained for the same spiked dilution in the absence of PK treatment. This may indicate that PK is degrading a good proportion of PrP^{Sc} as well as PrP^{c}. It is likely that the enzyme to substrate ratio is less with brain tissue than with spleen tissue due to much higher levels of PrP in brain compared with spleen tissue. As discussed in section 4.5 the problems of using PK to degrade PrP^{c} will always be relatively limited in sensitivity

but could be improved in this case by titrating the PK. Although values at 1.5 and 2.0 log dilutions lie above the cut-off their standard deviation which crosses the cut-off line makes these values not significant above the cut-off. Therefore the limit of detection for with PK treatment is in fact 1 log and is less than the same assay performed in the absence of PK.

The PrP^{Sc} present in vCJD spleen homogenate is detectable using these methods to at best 1.5 log dilutions of a 10% homogenate spiked into platelet poor plasma. In an attempt to provide a more accurate determination of assay sensitivity for spleen PrP^{Sc}, platelet poor plasma samples spiked with vCJD 10 % spleen homogenate were prepared in doubling dilutions between 1/10 and 1/5120. 900 µL samples of each dilution were assayed by CDI following NaPTA precipitation in the absence of PK treatment. The results of this assay are shown in Figure 53 below.

Figure 53



NaPTA concentration and CDI analysis of vCJD spleen homogenate spiked into human plasma. Mean ratios for each spiked plasma dilution are plotted and the error bars represent ±2 sd. The cut-off is the ratio ± 3 sd of unspiked 10 % spleen homogenate from a non-CJD neurological control.

The assay results shown in Figure 53 illustrate that PrP^{Sc} in vCJD spleen homogenate can be detected above cut-off to a limit of 1/640 dilution in platelet poor plasma, this is equivalcnt to a log dilution of $10^{-2.64}$.

This and the other vCJD spleen spiking assays described in this chapter confirm the suitability of NaPTA precipitation and the CDI assay technique for the detection of

PrP^{Sc} in spleen tissue. The levels of PrP^{Sc} in spleen tissue are in the order of 2-3 logs less than levels found in brain tissue. These differences reflect previously found differences in PrP^{Sc} levels between vCJD spleen and brain by western blotting studies [98,99]. These relative levels parallel the reported differences in infectivity between vCJD spleen and brain tissue as found in mice infectivity studies [100]. The results are consistent with PrP^{Sc} being the infectious agent, given that PrP^{Sc} remains in proportion with infectivity in these tissues, and also that the PrP^{Sc} found in spleen tissue is not physiologically different to that found in brain tissue.

7.4 *The detection of PrP^{Sc} in tonsil tissues of patients with vCJD*

To continue studies to detect the presence of PrP^{Sc} in the peripheral tissue of patients with vCJD, tonsil tissue was assayed using the CDI technique. 10 % homogenates of tonsil tissue in PBS + 1 % sarkosyl pH 7.4 from five patients with vCJD, four patients with sCJD and two non-CJD neurological controls were prepared. To 900 µL of each tonsil homogenate was added 100 µL of 20 % sarkosyl. Tissue homogenates samples were then pre-treated with PK at a final concentration of 50 µG/mL following those methods described in section 2.8.3. PK treated samples were then NaPTA precipitated and assayed by CDI following those methods described in sections 2.4.1 and 2.4.2. The results of this assay are shown in Table 23 below.

Table 23

Sample ID	Mean Ratio	2sd	Cut-off	Pos / Neg
vCJD				
RU 99/82	2.822	0.980	1.776	+
RU 99/90	36.046	4.086	1.776	+
RU 99/129	3.639	1.901	1.776	+
RU 00/25	4.536	2.006	1.776	+
RU 01/69	9.589	3.841	1.776	+
sCJD				
RU 96/153	1.101	0.341	1.776	-
RU 97/8	0.775	0.181	1.776	-
RU 97/15	1.016	0.136	1.776	-
RU 97/292	0.853	0.010	1.776	-
Ctrl				
Buffer ctrl	0.980	0.361	1.776	-
RU 99/20	1.264	0.022	1.776	-
RU 99/54	0.842	0.090	1.776	-
Pos Ctrl	256.641	226.766	1.776	+

Mean ratio of denatured sample / native sample europium cpm obtained by CDI analysis for tonsil samples obtained from 5 vCJD patients, 4 sCJD patients, 2 non-CJD neurological controls, a buffer control, and a positive control of 1/10 dilution of vCJD brain homogenate. The cut-off value was calculated from the mean ± 3 sd of non-CJD controls. All tonsil samples from patients with vCJD test positive for the presence of PrP^{Sc} above cut-off, all other samples except the positive control test negative.

The results from this CDI analysis confirm previous analysis of tonsil tissue from patients with vCJD and sCJD [99,162]. All vCJD tonsil samples test positive for the presence of PrP^{Sc} above a cut-off calculated from non-CJD neurological control samples, and all sCJD tonsil samples test negative for the presence of PrP^{Sc}. These results demonstrate the suitability of NaPTA precipitation and CDI analysis for the detection of low concentrations of PrP^{Sc} in tonsil tissue from patients with vCJD.

7.5 Detection of PrP^{Sc} in muscle tissue from vCJD and sCJD patients

Recent studies by Glatzel et al [163] have identified the presence of small quantities of PrP^{Sc} in both spleen and muscle tissue in a subset of Swiss patients who died of sCJD. This is the first report which details the presence of PrP^{Sc} outside the central nervous and olfactory systems in sCJD cases. Attempts to reproduce these findings in the National CJD Surveillance Unit have had limited success probably due to the reasons for variable results cited in the original publication, such as the non-homogenous distribution of PrP^{Sc} in muscle fibres and in distinct samples taken from the same tissue, and the fact that uncommon variants of sCJD with long duration are associated with those patients with extraneural PrP^{Sc}. Indeed the positive muscle sample was an atypical case with an unusually long disease duration.

NaPTA precipitation and CDI techniques were employed here to detect PrP^{Sc} in muscle tissue homogenates from patients with vCJD and sCJD. Muscle samples from 6 vCJD patients, 4 sCJD patients, and 2 non-CJD neurological controls were homogenised to 10 % in PBS + 1 % sarkosyl pH 7.4. Tissue homogenates were PK treated, NaPTA precipitated, and assayed by CDI following those methods described in sections 7.4, 2.4.1 and 2.4.2. The results of this assay are shown in table 24 below.

PrP^{Sc} was only detected in muscle tissue samples obtained from a single patient with sCJD. All remaining muscle tissue homogenates from patients with sCJD and vCJD tested negative for the presence of PrP^{Sc} against a cut-off provided by the CDI ratios of non-CJD neurological control muscle samples. The positive sCJD muscle sample had previously tested positive by western blot analysis following methods of Glatzel et al [163] (personal communication Dr A Peden, National CJD Surveillance Unit).

Table 24

Sample ID	Mean Ratio	2sd	Cut-off	Pos / Neg
vCJD				
RU 99/82	1.241	0.156	1.5467	-
RU 99/90	0.994	0.150	1.5467	-
RU 99/129	1.223	0.372	1.5467	-
RU 00/25	0.837	0.099	1.5467	-
RU 00/101	0.962	0.253	1.5467	-
RU 01/69	1.001	0.342	1.5467	-
sCJD				
RU 96/153	1.123	0.338	1.5467	-
RU 97/8	0.920	0.178	1.5467	-
RU 97/15	1.114	0.311	1.5467	-
RU 97/292	8.438	24.959	1.5467	+
Ctrl				
RU 99/54	1.078	0.392	1.5467	-
RU 98/145	0.829	0.228	1.5467	-
Pos Ctrl	129.411	129.411	1.5467	+

Mean ratio of denatured / native europium cpm obtained by CDI analysis for muscle samples obtained from 6 vCJD patients, 4 sCJD patients, 2 non-CJD neurological controls, and a positive control of 1/10 dilution of vCJD brain homogenate. The cut-off value calculated from the mean ± 3 sd of non-CJD controls.

To verify the result of the detection of PrP^{Sc} in this particular sCJD muscle sample, the tissue was re-sampled and assayed as above alongside a non-CJD neurological control muscle sample and a positive vCJD brain control. In this assay (data not shown) the sample did not test positive for PrP^{Sc} above the cut-off. This illustrates the likely non-homogeneous distribution of PrP^{Sc} in muscle tissue, and the difficulties of verifying positivity with different samplings from the same tissue. The lack of significant numbers of muscle samples which test positive for PrP^{Sc} are possibly a consequence of the small number of samples tested. It may also be

because in CDI analysis NaPTA precipitated samples are resuspended in larger volumes and assayed in triplicate, whereas in western blot analysis the PrP^{Sc} if present in a sample is more concentrated and is run on a single lane of a western blot.

In this chapter NaPTA precipitation and CDI assay techniques have been used to detect PrP^{Sc} in spleen, tonsil, and muscle tissue homogenates from patients with vCJD, sCJD, and non-CJD neurological controls. PrP^{Sc} can be readily detected in spleen and tonsil tissue homogenates from patients with vCJD using these methods. PrP^{Sc} was undetected in tonsil samples from patients with sCJD, but could be detected in a sample of muscle tissue. These studies verify that CDI is suitable for the detection of PrP^{Sc} in peripheral tissues. It does not appear that any physiological differences that may exist between the type of PrP^{Sc} present in brain and peripheral tissue restrict the ability for its detection using these methods. Indeed it suggests that the PrP^{Sc} present in both brain and peripheral tissue is the same component and that there do not appear to be any differences in form, presentation, protease sensitivity or epitope presentation which affect the methods used here for its detection. Considering that PrP^{Sc} present in peripheral tissue most likely originates from peripheral blood this methodology may with further refinements of sensitivity be suitable for the detection of PrP^{Sc} in the peripheral blood of patients with vCJD.

Chapter 8: Atomic Dieletric Resonance (ADR) spectroscopy analysis of blood samples from patients with vCJD and sCJD.

8.1 Background

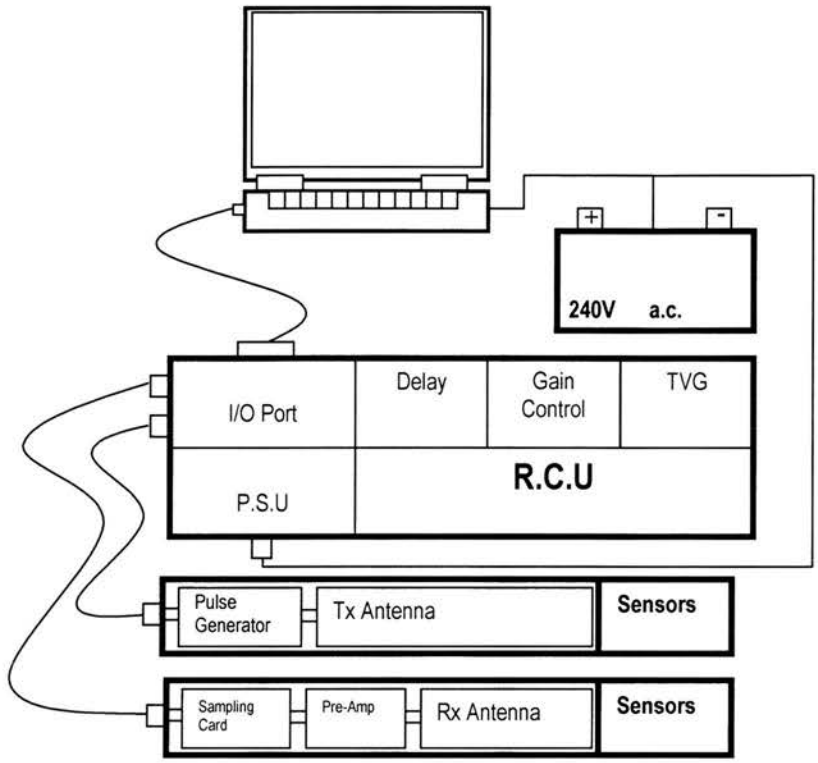
An opportunity arose to employ ADR spectroscopy techniques in an analysis of clinical CJD whole blood samples. This technology is distinct from other assays presented in this thesis because the distinction of CJD samples from control samples is not based upon the detection of PrP^{Sc} but of the ADR of undefined markers. ADR is proprietary and patented technology owned and developed by Radar World Ltd, Edinburgh. ADR has actual or potential use in a wide range of applications including satellite and subsurface imaging, statistical classification techniques, geological and biological material identification, and veterinary and medical applications. It is derived from specialised RADAR used in satellite imaging and surveillance. ADR has remarkable subsurface imaging properties, used mainly in geological, engineering and archaeological investigations. ADR can also be applied to materials recognition. Materials have unique energy absorption and reflection properties and provide spectra in the broad range of frequencies employed that provide signatures for recognition and matching. Related materials will match closely and matches can be ranked. As yet, only some of the spectral peaks can be attributed to constituents of complex materials such as the presence of certain elements, dielectric constants however can be measured very accurately. The power output is very low and the technology has been approved as a medical device and some trials have been undertaken and are planned in imaging contexts. In materials

analysis it does not consume sample and is essentially non-destructive, and there are also no reagents involved.

8.2 ADR scanning apparatus

The apparatus used for the ADR spectral analysis was composed of the following hardware components: a radar control unit (RCI), a pulse generator, and a sample chamber incorporating the transmission and receiving antennae. These components are illustrated in Figure 54 below.

Figure 54



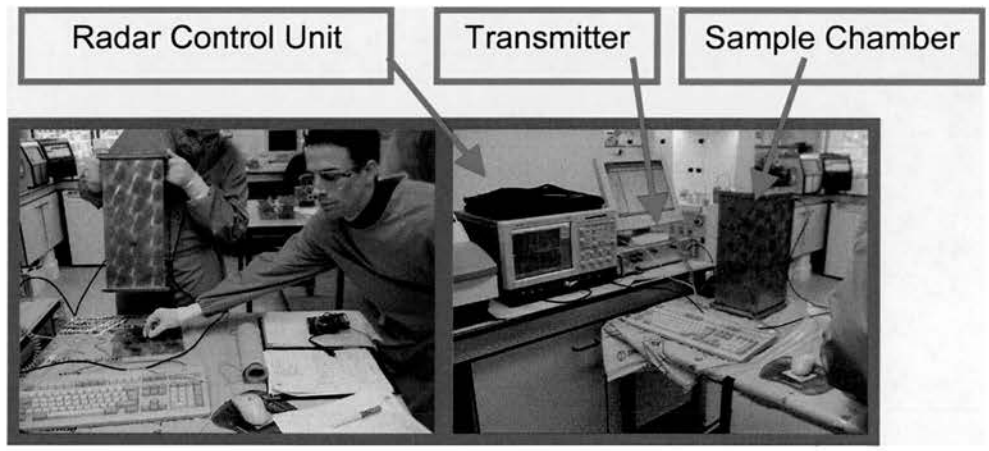
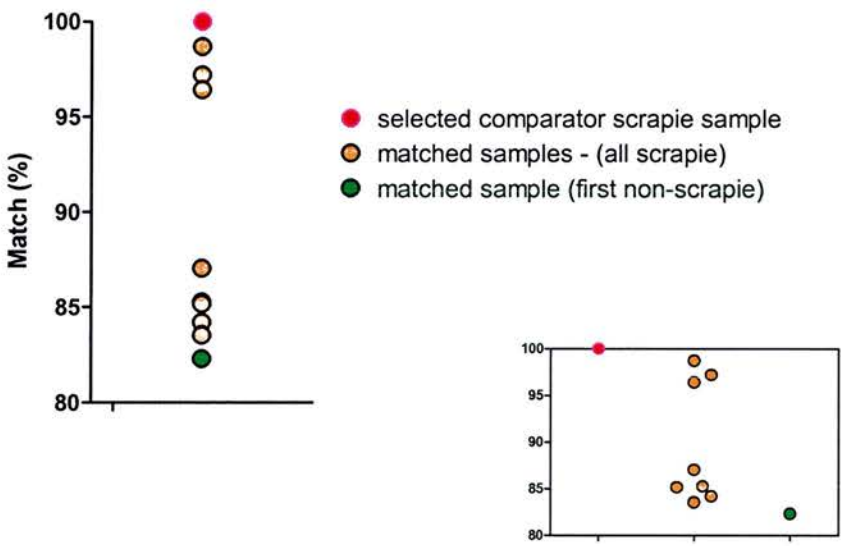


Diagram and photographs to show the components of the radar analysis hardware.

In an early study in collaboration with the Institute of Animal Health, Edinburgh, slides of brain sections from experimental scrapie infected mice and uninfected controls were scanned by ADR. It was possible to discriminate scrapie-infected mice from the normal controls by simple match ranking of their ADR spectra (see section 8.4.2) as shown in Figure 55 below.

Figure 55



Mouse brain samples (histology slides)-best 10 matches using ADR rank matching methods

These results were reported in a poster session at the International Conference on Transmissible Spongiform Encephalopathies, Edinburgh 2002. This spectroscopic methodology was given further credence by the report of the use of Fourier-

transformed Infrared spectroscopy for the discrimination between BSE-infected and non-BSE cattle sera [141].

To consider whether ADR scanning may be of use in the development of a peripheral blood screening test for CJD the following two experimental studies were carried out.

8.3 First experiment study design.

Clinical whole blood samples from patients with vCJD, sCJD, and non-CJD neurological controls were obtained from the National CJD Surveillance Unit. Healthy adult whole blood samples were obtained from the Scottish National Blood Transfusion Service, Edinburgh. In addition a series of aliquots of healthy adult whole blood were spiked to a 1/10 dilution with 10 % brain homogenates prepared from brain tissue obtained from vCJD, sCJD, a non CJD neurological control patient with Alzheimer's disease, 263K scrapie infected hamster brain homogenate or healthy hamster brain homogenate. Unspiked control whole blood was included as a control. There were therefore 10 classes of blood samples coded A to J, consisting of 5 samples in each group, 50 samples total. The sample group codes are shown in Table 25 below.

Table 25

CLINICAL blood samples	
Sample	***Description***
A	vCJD
B	sCJD
C	Neurological
D	Healthy adult

Blood SPIKED with brain material	
Sample	***Description***
E	Unspiked normal blood
F	Alzheimer's
G	vCJD
H	sCJD
I	263k hamster
J	Normal hamster

Clinical and spiked sample group codes A-J for the first ADR spectral analysis

Of the 5 samples in each group 2/5 were un-blinded as controls for training, leaving the remaining 3/5 samples as blind test samples which were randomised. 4 scans of each sample were taken, so that for training there were 20 known samples (4 scans per sample = 80 scans), and for the blind tests there were 30 samples (4 scans per sample = 120 scans).

For the clinical series A-D each class is composed of 5 different individual patient samples. Samples were retrieved from frozen archived material stored by the National CJD Surveillance Unit. Samples E-J represent the spiking study, the same blood was used throughout E-J and was frozen before use to give it the same treatment as archived clinical material. All samples (0.5 mL) were dispensed in the same batch of 2 mL polypropylene tubes with caps and readings were also taken on empty tubes for subtraction of spectra. As far as we could ensure, no artefacts were introduced from any differences in handling, storage, or dispensing samples.

All tubes were vortexed for even dispersal of contents immediately before placing in the ADR chamber for scanning. Sample codes were recorded with each ADR spectra stored in the linked data acquisition computer. ADR scanning was performed by Dr Colin Stove and Mr Michael Robinson from Radar World Ltd.

8.4 Analysis of ADR spectra.

The ADR spectral data generated for clinical and spiked blood samples was analysed in several stages using Radamatic ADR and image analysis software developed by Radar World. Firstly un-blinded training clinical blood samples of vCJD and sCJD were studied to determine whether there were any predominant spectral features which could be used to distinguish the two groups.

8.4.1 Primary analysis

Using Radamatic ADR software, spectral reflections for a single vCJD and sCJD sample were analysed. The data generated by scanning an empty 2mL storage tube was firstly subtracted from the spectral data generated for the vCJD and sCJD samples so that the resultant data represented the reflections in blood samples alone with no contribution from the sample tube. Data was complied into tables which show ADR parameters (f1, and f2) between 1 - 20 % and between 90 - 99 % energy bins (E-bin) and the ADR-ratio which is a measure of the rate of change of resonance with frequency cut-off per energy bin. This data is shown for vCJD and sCJD samples in Table 26 below.

Table 26

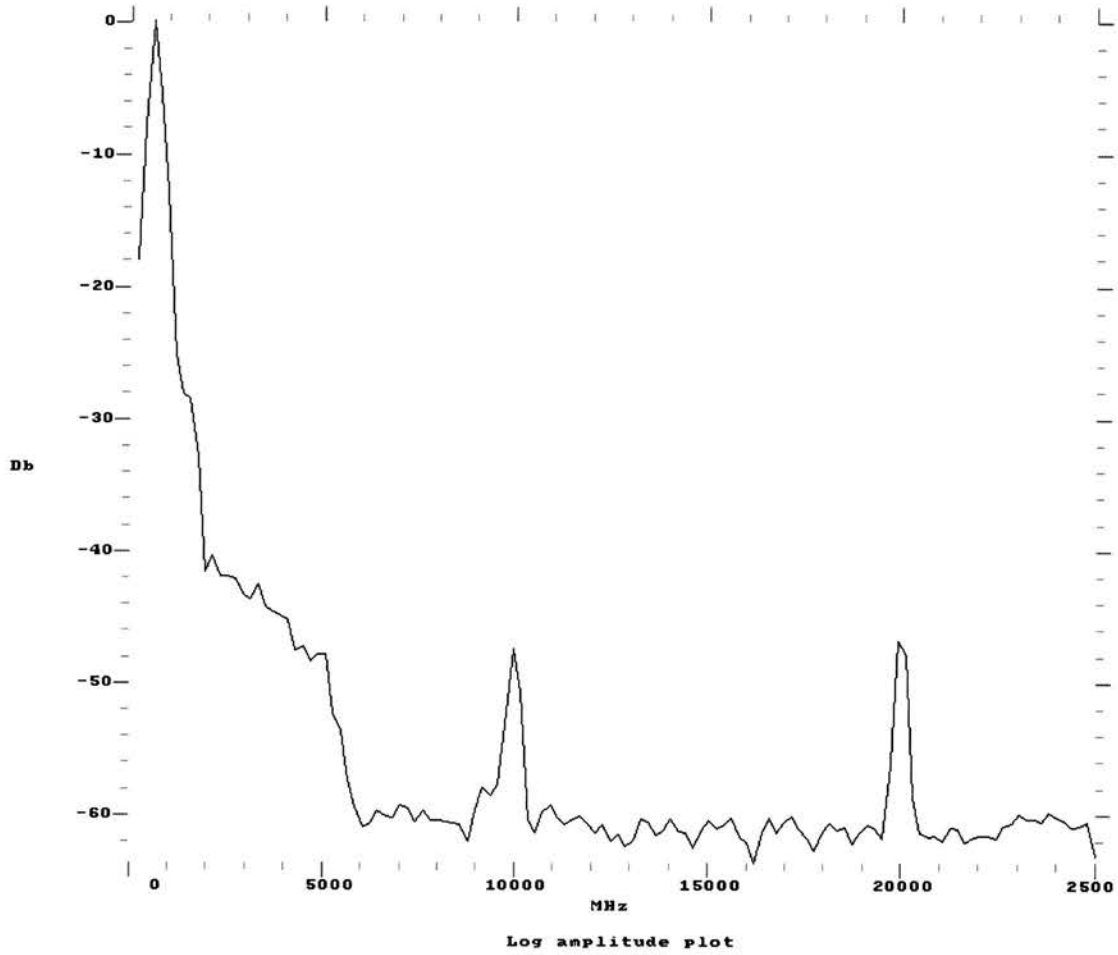
E(%)	f1(MHz)		f2(MHz)		ADR-Ratio	
	vCJD	sCJD	vCJD	sCJD	vCJD	sCJD
1.0	186	22	42.0	1.4	4.412	15.700
2.0	212	45	17.8	2.9	11.939	15.700
3.0	230	67	7.4	4.3	31.063	15.700
4.0	248	90	5.0	5.7	49.842	15.700
5.0	266	112	8.1	7.1	32.733	15.700
6.0	284	134	13.4	8.6	21.246	15.700
7.0	302	157	19.1	10.0	15.858	15.700
8.0	321	179	24.2	11.0	13.264	16.292
9.0	339	199	25.6	10.2	13.229	19.478
10.0	357	214	24.0	10.9	14.851	19.674
11.0	375	229	20.4	11.0	18.373	18.048
12.0	391	245	15.6	14.7	25.022	16.605
13.0	394	260	10.9	16.9	36.211	15.393
14.0	397	275	7.4	19.1	53.853	14.390
15.0	400	290	6.0	21.4	66.388	13.559
16.0	403	305	6.1	23.7	66.204	12.866
17.0	406	320	6.1	25.9	66.019	12.351
18.0	409	335	6.2	26.7	65.832	12.516
19.0	412	350	6.3	27.1	65.644	12.887
20.0	415	365	6.3	27.2	65.456	13.394
90.0	717	904	22.5	6.1	31.810	147.013
91.0	727	915	21.8	5.6	33.446	162.748
92.0	738	927	22.3	5.1	33.075	181.498
93.0	748	938	24.1	4.6	31.050	204.118
94.0	759	950	28.2	4.1	26.890	231.745
95.0	769	961	34.6	3.6	22.233	265.856
96.0	780	973	42.9	5.6	18.170	174.787
97.0	832	1054	51.2	26.7	16.253	39.452
98.0	889	1173	48.0	22.1	18.539	53.040
99.0	947	1320	32.3	13.6	29.348	97.216

Data shows the ADR parameters f1 and f2 and the ADR-ratio for individual vCJD and sCJD samples for energy levels E-bin 1 % to E-bin 20 %, and E-bin 90 % to E-bin 99 %.

There are a number of important observations which can be made from studying the data presented in Table 26 which summarises the 1st 20 % E-bin results and the last 10 % E-bin results for vCJD and sCJD from the detailed fast Fourier transform (FFT) tables computed by the Radamatic software. At the 99 % energy level the frequency resonance is 947 MHz for vCJD compared with 1320 MHz for sCJD. However the actual resonance value at this level as computed by the standard variance f2 ADR parameter statistic is much higher (+/-32.3 MHz) for vCJD than that (+/-13.6 MHz) for sCJD. This is a significant result, which can be summarised in the ADR-Ratio. This is an inverse index and the lower the ADR-Ratio the higher the ADR parameter. In this case vCJD yields an ADR-ratio of 29.348 at the 99 % energy-bin compared with 97.216 for sCJD. There is also a distinct trend of resonance variability with vCJD compared with sCJD. For the 1st 7 energy-bins the ADR-ratio for sCJD is constant at 15.7, whereas the ADR-ratios for vCJD vary considerably from 4 (1%), 11 (2%), 31 (3%), 49 (4%), 32 (5%), 21 (6%) and 15 (7%).

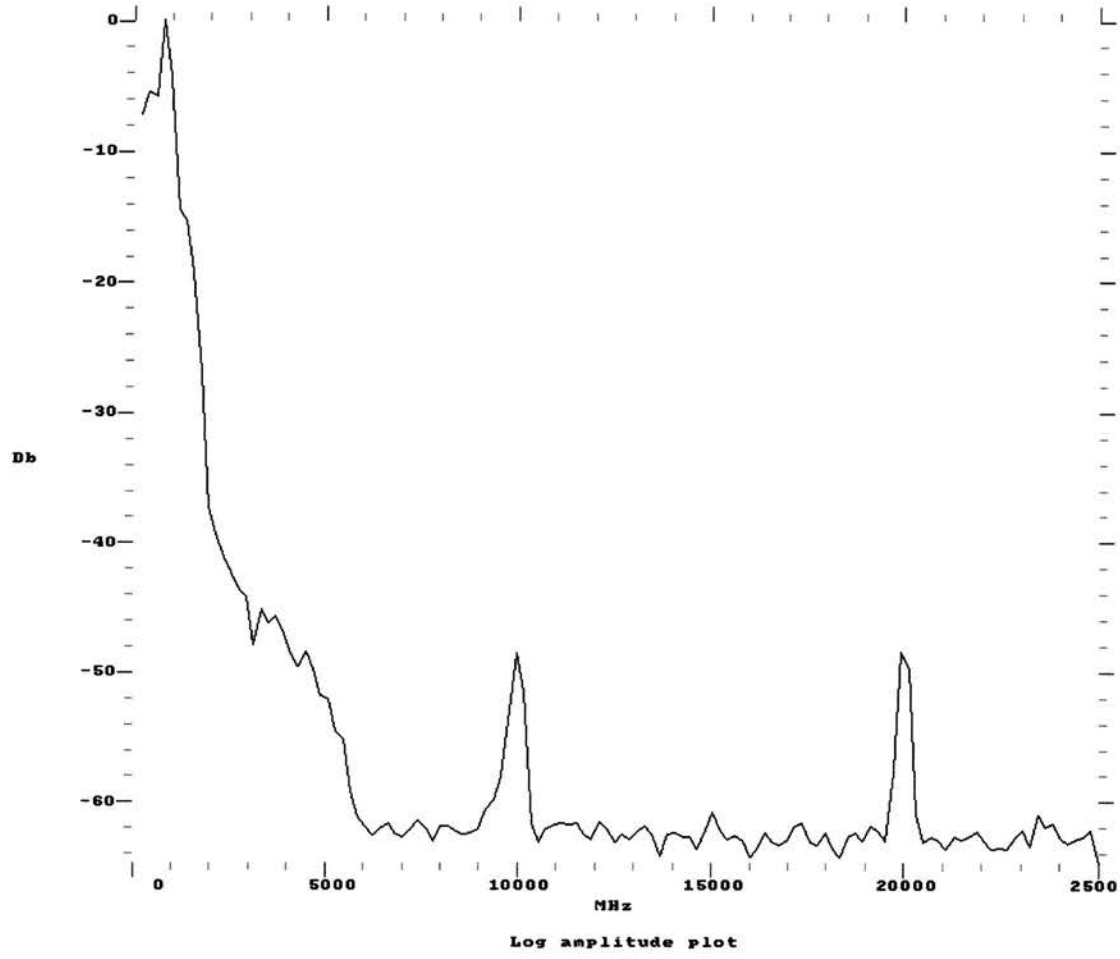
To highlight spectral differences between samples, the amplitude (or maximum wave displacement of absorption spectra) of the same vCJD and sCJD samples was plotted against energy in decibels (Db). Log amplitude plots are shown for vCJD and sCJD in Figures 56 and 57 below. Figure 58 shows the FFT difference plot created by subtracting the vCJD spectral data from the sCJD spectral data. This illustrates the variability of vCJD over sCJD, particularly from 3000 MHz to 25000 MHz and could be a useful diagnostic indicator of differences between groups.

Figure 56



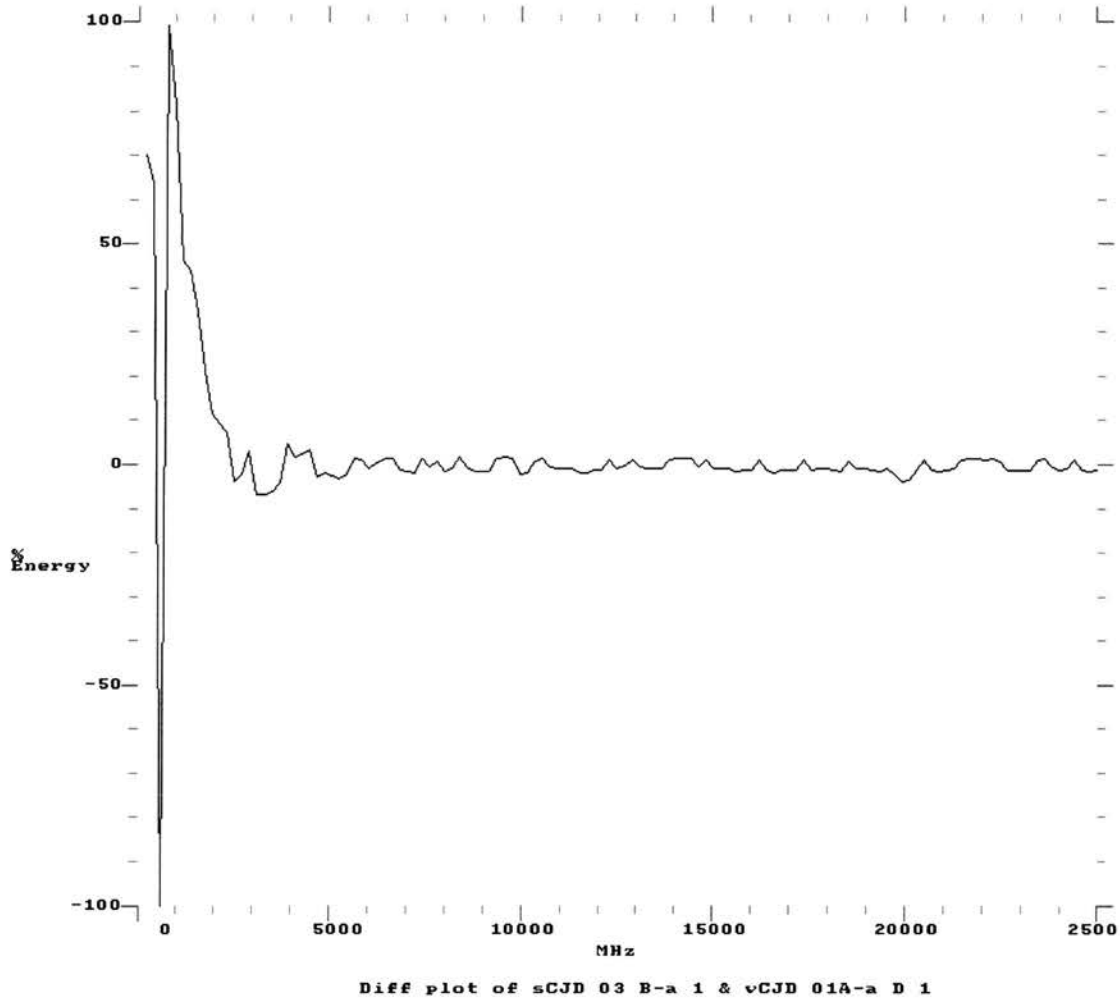
Log Amplitude Plot of Absorption Spectra for vCJD sample ID01 A1-a minus ET-04 Chamber and sample container effects

Figure 57



Log Amplitude Plot of Absorption Spectra for sCJD sample ID03 B1-b minus ET-04 Chamber and sample container effects

Figure 58



Illustrates the FFT Difference plot by subtracting the vCJD spectra from the sCJD spectra. This shows the variability of vCJD over sCJD, particularly from 3000MHz to 25000MHz and is again a useful diagnostic indicator of differences.

It is not apparent at this stage of the analysis whether these interesting spectral differences between individual samples are associated with the clinical class of the samples. They may merely reflect differences such as blood type between individuals and factors which are not associated with disease status. A detailed analysis of much larger sample numbers from each clinical class may permit the identification of particular spectral patterns able to distinguish vCJD from sCJD or from healthy adult blood samples. Infraraed spectral analysis and pattern recognition analysis

techniques have been used in a similar way by Schmitt et al [140] to distinguish sera from 263K scrapie infected hamsters from healthy control animals, and by Lasch et al [141] to distinguish BSE serum samples from non-BSE controls. These methods detect characteristic signatures originating from the various biomolecules present in sera which distinguish groups without the need to detect PrP^{Sc}. These studies needed very large sample numbers, and a simple two-way classification before they could identify discriminatory spectral properties. Here we are using very small numbers of samples and several sample categories by comparison.

8.4.2 Match ranking analysis.

In an attempt to identify blind samples, unprocessed Fourier transformed ADR spectra were match ranked. This method employed a single scan of a known training sample used as a reference spectrum against which the remaining scans of control and test samples were matched. The closeness of the matching was based upon determining the correlation coefficient values and setting them in order, therefore the closer to 1 or in this case 100 % the more exact the match. Three samples, Test 44 (D5 – clinical, healthy control), Test 45 (E – spike control, unspiked control blood), and Test 31 (J – spiked, normal hamster brain) were rejected from the analysis because of bad replication, apparent through the spread of replicate scans on match ranking. It is not know why this occurred, one suggestion is that the vortexing immediately before scanning introduced features, for example currents and bubbles, which were still stabilising during the relatively rapid scanning. This should be investigated, and might be avoided by procedural and design modifications.

The inclusion of the spiking study in the analysis of the clinical study made analysis complicated due to the number of different class variables. To simplify the analysis blind samples from the spiking study were excluded. The identity of blind samples and controls are shown below in Table 27.

Table 27

A

Sample no.	Class
1	A1
2	A2
3	B1
4	B2
5	C1
6	C2
7	D1
8	D2
9	E
10	E
11	F
12	F
13	G
14	G
15	H
16	H
17	I
18	I
19	J
20	J
21	A3
22	B3
23	C3
24	D3
25	E
26	F
27	G
28	H
29	I
30	J
31	J
32	I
33	H
34	G
35	F
36	E
37	D4
38	C4
39	B4
40	A4
41	A5
42	B5
43	C5
44	D5
45	E
46	F
47	G
48	H
49	I
50	J

B

A	vCJD
B	sCJD
C	neuro ctrl
D	apheresis donors
E	unspiked
F	alzheimer spiked
G	vCJD spiked
H	sCJD spiked
I	263k spiked
J	ctrl hamster spiked

C

Training samples
Blind samples

	Sample codes
A1	1553
A2	1532
A3	1518
A4	1509
A5	1476
B1	1588
B2	1572
B3	1540
B4	1551
B5	1542
C1	1274
C2	1272
C3	1243
C4	1223
C5	1180
D1	27
D2	28
D3	29
D4	30
D5	31

(A,B) Sample class identities for control and blind samples. (C)Sample codes for clinical samples allow identification of sample from the National CJD Surveillance Unit archives.

Table 28

Radar World

COMPARISON OF SAMPLE A1-s1mET4 TO CONTROL DATA SET 13 (A+B+C+D+E+F+G+H+I+J) & BLIND TEST DATA SET

	A1mET4	1		
No	Name	Sample	Dist.	
1	A1mET4	2	0.032094	99.92%
2	A1mET4	3	0.20358	99.46%
3	T9-41	1	0.250892	99.34%
4	T9-41	3	0.251589	99.33%
5	T9-41	4	0.251724	99.33%
6	T9-41	2	0.252874	99.33%
7	T11-43	1	0.304431	99.20%
8	T11-43	2	0.306708	99.19%
9	T8-40	1	0.309992	99.18%
10	T8-40	3	0.310788	99.18%
11	T8-40	2	0.310859	99.18%
12	T8-40	4	0.311027	99.18%
13	T10-42	3	0.315265	99.17%
14	T10-42	2	0.316742	99.16%
15	T11-43	3	0.31727	99.16%
16	T10-42	4	0.317705	99.16%
17	T10-42	1	0.317845	99.16%
18	T11-43	4	0.327323	99.13%
19	T6-38	4	0.374821	99.01%
20	T7-39	4	0.374821	99.01%
21	T7-39	3	0.377015	99.00%
22	T7-39	1	0.379169	99.00%
23	T7-39	2	0.38055	98.99%
24	B1mET4	1	0.393229	98.96%
25	B1mET4	2	0.432744	98.86%
26	B1mET4	3	0.435285	98.85%
27	G1mET4	4	0.441666	98.83%
28	G1mET4	3	0.445271	98.82%
29	B1mET4	4	0.45304	98.80%
30	G1mET4	2	0.456411	98.79%
31	F1mET4	1	0.480719	98.73%
32	F1mET4	2	0.484311	98.72%
33	C1mET4	2	0.489689	98.70%
34	H1mET4	4	0.49103	98.70%
35	T6-38	1	0.492239	98.70%
36	T6-38	3	0.49502	98.69%
37	H1mET4	2	0.495057	98.69%
38	C1mET4	1	0.495087	98.69%
39	T6-38	2	0.496163	98.69%
40	H1mET4	3	0.496705	98.69%
41	F1mET4	4	0.497029	98.69%
42	F1mET4	3	0.499586	98.68%
43	C1mET4	3	0.506293	98.66%
44	H1mET4	1	0.506928	98.66%
45	C1mET4	4	0.515813	98.64%
46	D1mET4	1	0.515917	98.64%
47	T1-21	1	0.516305	98.63%
48	D1mET4	2	0.51775	98.63%
49	D1mET4	3	0.520766	98.62%
50	T1-21	2	0.520938	98.62%
51	T2-22	1	0.52678	98.61%
52	D1mET4	4	0.526902	98.61%
53	T2-22	2	0.527469	98.61%
54	T2-22	4	0.527591	98.60%
55	T2-22	3	0.527947	98.60%
56	T1-21	3	0.528874	98.60%
57	J1mET4	3	0.531987	98.59%
58	G1mET4	1	0.532426	98.59%
59	J1mET4	4	0.533054	98.59%
60	J1mET4	2	0.533095	98.59%
61	J1mET4	1	0.533367	98.59%
62	E1mET4	1	0.535264	98.58%
63	E1mET4	2	0.53763	98.58%
64	T1-21	4	0.539702	98.57%
65	J1mET4	4	0.540513	98.57%
66	J1mET4	1	0.541323	98.57%
67	E1mET4	3	0.543002	98.56%
68	J1mET4	2	0.543481	98.56%
69	E1mET4	4	0.543646	98.56%
70	A1mET4	4	0.544193	98.56%
71	T4-24	1	0.545318	98.56%
72	T3-23	4	0.545754	98.56%
73	T3-23	3	0.545991	98.56%
74	J1mET4	3	0.546125	98.56%
75	T4-24	3	0.546492	98.55%
76	T4-24	4	0.546683	98.55%
77	T4-24	2	0.547065	98.55%
78	T3-23	2	0.547166	98.55%
79	T3-23	1	0.547238	98.55%
80	T5-37	4	0.549521	98.55%
81	T5-37	1	0.549726	98.55%
82	T5-37	3	0.549892	98.55%
83	T5-37	2	0.549988	98.55%

CTRL	CLASSES	COLOUR	%
vCJD	A		99.3120%
sCJD	B		98.8663%
Neuro.	C		98.6725%
Aph.Dons	D		98.6233%
GRB norm	E		98.5720%
E+ALZ	F		98.7025%
E+vCJD	G		98.7598%
E+sCJD	H		98.6840%
E+263k	I		98.5638%
E+HAMc	J		98.5905%
	T1-21		98.6073%
	T2-22		98.6045%
	T3-23		98.5543%
	T4-24		98.5545%
	T5-37		98.5455%
	T6-38		98.7708%
	T7-39		99.0003%
	T8-40		99.1783%
	T9-41		99.3338%
	T10-42		99.1618%
	T11-43		99.1698%

RANK	CLASS	COLOUR	%
1	T9-41		99.3338%
2	A		99.3120%
3	T8-40		99.1783%
4	T11-43		99.1698%
5	T10-42		99.1618%
6	T7-39		99.0003%
7	B		98.8663%
8	T6-38		98.7708%
9	G		98.7598%
10	F		98.7025%
11	H		98.6840%
12	C		98.6725%
13	D		98.6233%
14	T1-21		98.6073%
15	T2-22		98.6045%
16	J		98.5905%
17	E		98.5720%
18	I		98.5638%
19	T4-24		98.5545%
20	T3-23		98.5543%
21	T5-37		98.5455%

Match ranking table of all sample classes including blind samples from clinical classes A-D. Samples are ranked using a scan of a vCJD control as reference sample. The colour key for sample class, and a table showing the top ranks are included on the right of the Figure.

An example of a matching rank table is shown in Table 28. A vCJD sample scan was selected as the reference sample for matching. The class letter and colour identify the known training samples. The second set of training samples A2-J2 were excluded from this matching table (saved for a later validation), as were the blind samples from the spiked classes E-J. Therefore in the matching table above are 4 scans of each of the first training samples from classes A-J alongside the unknown blind samples from classes A-D.

There was discrete clustering of some of the samples according to class, which indicated some strong clinical features of many samples which dominated over the individuality of the control blood sample donors, since 2/3 vCJD samples (class A) and 3/4 sCJD samples (class B) were in the top 6 of the matches to A1, while the normal adult blood samples were mostly located at the bottom end of the matching ranks as being least like the vCJD sample A1. Identification of unknowns through match ranking was not just a case of looking at a single match rank table, but involved looking at the behaviour of the test sample against other known samples as a base for matching. The spread of rank match identities in the whole sample population (100 % - 98.5 %) is close showing that samples share a great deal of similarity compared with the range for the mouse scrapie match ranking of histology slides where the rank matching values ranged from 100 % to 82 % for the different histology slides.

In Table 29 a match-ranking table is shown in which a normal donor sample spiking control, E was used as the reference for matching.

Table 29

Radar World

0	E1mET4	3		
No	Name	Sample	Dist.	%
1	E1mET4	2	0.000087	100.00%
2	E1mET4	1	0.000195	100.00%
3	E1mET4	4	0.000224	100.00%
4	D1mET4	3	0.000689	99.99%
5	D1mET4	2	0.000860	99.99%
6	D1mET4	1	0.000938	99.99%
7	T4-24	1	0.003963	99.96%
8	I1mET4	4	0.004495	99.95%
9	T4-24	2	0.004847	99.95%
10	J1mET4	2	0.004978	99.95%
11	J1mET4	1	0.005226	99.95%
12	J1mET4	4	0.005876	99.94%
13	C1mET4	4	0.005886	99.94%
14	J1mET4	3	0.005921	99.94%
15	T4-24	3	0.005955	99.94%
16	F1mET4	3	0.006402	99.93%
17	F1mET4	1	0.006487	99.93%
18	F1mET4	2	0.006572	99.93%
19	I1mET4	1	0.006801	99.93%
20	T4-24	4	0.007097	99.93%
21	I1mET4	2	0.007538	99.92%
22	T3-23	4	0.007579	99.92%
23	T3-23	3	0.007755	99.92%
24	C1mET4	2	0.008046	99.92%
25	T3-23	2	0.008140	99.92%
26	I1mET4	3	0.008485	99.91%
27	T3-23	1	0.008682	99.91%
28	C1mET4	3	0.008938	99.91%
29	F1mET4	4	0.008958	99.91%
30	C1mET4	1	0.009198	99.91%
31	T2-22	1	0.011736	99.88%
32	T2-22	4	0.012758	99.87%
33	T2-22	2	0.012959	99.87%
34	T2-22	3	0.013262	99.86%
35	H1mET4	1	0.019241	99.80%
36	H1mET4	4	0.020459	99.79%
37	H1mET4	2	0.020901	99.79%
38	H1mET4	3	0.021220	99.78%
39	T1-21	4	0.023994	99.75%
40	B1mET4	4	0.024062	99.75%
41	T1-21	3	0.026774	99.73%
42	T1-21	2	0.027626	99.72%
43	T1-21	1	0.029949	99.69%
44	B1mET4	3	0.032997	99.66%
45	T5-37	1	0.033756	99.65%
46	T5-37	4	0.033825	99.65%
47	T5-37	2	0.034017	99.65%
48	T5-37	3	0.034244	99.65%
49	B1mET4	2	0.035882	99.63%
50	B1mET4	1	0.052606	99.46%
51	A1mET4	4	0.107274	98.90%
52	A1mET4	3	0.178333	98.17%
53	T6-38	2	0.214793	97.79%
54	T6-38	3	0.215055	97.79%
55	T6-38	1	0.221168	97.73%
57	T7-39	1	0.257028	97.36%
58	G1mET4	2	0.257412	97.35%
59	T7-39	2	0.258854	97.34%
60	G1mET4	3	0.260111	97.32%
61	G1mET4	4	0.265300	97.27%
62	T7-39	3	0.268383	97.24%
63	T11-43	1	0.269600	97.23%
64	T11-43	2	0.271049	97.21%
65	T11-43	3	0.272931	97.19%
66	T11-43	4	0.274540	97.18%
67	T6-39	4	0.281850	97.10%
68	T7-39	4	0.281850	97.10%
69	T8-40	1	0.300316	96.91%
70	T8-40	4	0.300499	96.91%
71	T8-40	3	0.301854	96.90%
72	T8-40	2	0.302718	96.89%
73	T10-42	3	0.304834	96.86%
74	T10-42	1	0.306779	96.84%
75	T10-42	4	0.307093	96.84%
76	T10-42	2	0.307821	96.83%
77	T9-41	3	0.315264	96.76%
78	T9-41	4	0.315754	96.75%
79	T9-41	2	0.316873	96.74%
80	T9-41	1	0.317068	96.74%
81	A1mET4	2	0.386593	96.02%
82	A1mET4	1	0.543002	94.41%

CTRL	CLASSES	COLOUR
vCJD	A	
sCJD	B	
Neuro.	C	
Healthy	D	
GRB norm	E	
E+ALZ	F	
E+vCJD	G	
E+sCJD	H	
E+263k	I	
E+HAMc	J	

Matching a healthy donor (single replicate E1mET4) to other known and blind test replicate scans including all the unknown clinical study samples and the control samples from the spiking study group.

Normal donor samples (class D) were most like the reference sample (unspiked control blood) while vCJD and sCJD samples (classes A and B) were least like it. These were essentially the same samples which lay at opposite ends of the ranking shown where a vCJD sample was used as the reference in Table 28. The CJD spiked sample classes tended to be located at the clinical CJD end of the rank while the Alzheimer's and control samples tended to lie at the normal end of the rank. However, only in the extreme end ranks of these series in either table could the blind test samples be identified with any confidence.

To simplify the matching tables the known control spiked samples were removed so leaving a rank matching of known and blind samples from classes A-D and the class E control of unspiked healthy adult whole blood. This rank-matching table demonstrates the suitability of this method to segregate vCJD, sCJD, neurological control non-CJD, and healthy adult donor whole blood samples. This match ranking is illustrated in Table 30 below.

Table 30

Radar world

MATCHING TABLE FOR A1-s1mET4

No	Name	Sample	Dist.	%	Mean A%	Mean B%	Mean C%	Mean D%	Mean E%
1	A1mET4	2	0.032094	99.9150%	99.92%				
2	A1mET4	3	0.20358	99.4610%					
3	T9-41	1	0.250892	99.3360%					
4	T9-41	3	0.251589	99.3340%					
5	T9-41	4	0.251724	99.3340%					
6	T9-41	2	0.252874	99.3310%	99.33%				
7	T11-43	1	0.304431	99.1950%			99.20%		
8	T12-44	3	0.305545	98.9460%				98.95%	
9	T11-43	2	0.306708	99.1890%			99.19%		
10	T12-44	1	0.307082	98.9410%				98.94%	
11	T8-40	1	0.309992	99.1800%	99.18%				
12	T8-40	3	0.310788	99.1780%					
13	T8-40	2	0.310859	99.1780%					
14	T8-40	4	0.311027	99.1770%	99.18%				
15	T10-42	3	0.315265	99.1660%		99.17%			
16	T12-44	2	0.316035	98.9100%				98.9100%	
17	T10-42	2	0.316742	99.1620%		99.16%			
18	T11-43	3	0.31727	99.1610%					
19	T10-42	4	0.317705	99.1600%					
20	T10-42	1	0.317845	99.1590%		**99.16%**			
21	T11-43	4	0.327323	99.1340%					
22	T6-38	4	0.374821	99.0080%			99.01%		
23	T7-39	4	0.374821	99.0080%		99.00%			
24	T7-39	3	0.377015	99.0030%					
25	T7-39	1	0.379169	98.9970%					
26	T7-39	2	0.38055	98.9930%					
27	B1mET4	1	0.393229	98.9600%					
28	T12-44	4	0.399565	98.6220%				98.95%	
29	B1mET4	2	0.432744	98.8550%					
30	B1mET4	3	0.435285	98.8480%					
31	B1mET4	4	0.45304	98.8020%		98.80%			
32	C1mET4	2	0.489889	98.7040%			98.70%		
33	T6-38	1	0.492239	98.6980%					
34	T6-38	3	0.49502	98.6900%					
35	C1mET4	1	0.495087	98.6900%					
36	T6-38	2	0.496163	98.6870%					
37	C1mET4	3	0.506293	98.6610%					
38	C1mET4	4	0.515813	98.6350%			98.67%		
39	D1mET4	1	0.515917	98.6350%				98.64%	
40	T1-21	1	0.516305	98.6340%	98.63%				
41	D1mET4	2	0.51775	98.6300%					
42	D1mET4	3	0.520766	98.6220%				98.62%	
43	T1-21	2	0.520938	98.6220%	98.62%				
44	T2-22	1	0.52678	98.6060%					
45	T2-22	2	0.527469	98.6050%					
46	T2-22	4	0.527591	98.6040%					
47	T2-22	3	0.527947	98.6030%		**98.60%**			
48	T1-21	3	0.528874	98.6010%					
49	E1mET4	1	0.535264	98.5840%					98.58%
50	E1mET4	2	0.53763	98.5780%					
51	T1-21	4	0.539702	98.5720%	98.61%				
52	E1mET4	3	0.543002	98.5640%					
53	E1mET4	4	0.543646	98.5620%					98.57%
54	T4-24	1	0.545318	98.5570%				98.56%	
55	T3-23	4	0.545754	98.5560%					
56	T3-23	3	0.545991	98.5560%					
57	T4-24	3	0.546492	98.5540%					
58	T4-24	4	0.546683	98.5540%					
59	T4-24	2	0.547065	98.5530%				98.55%	
60	T3-23	2	0.547166	98.5530%					
61	T3-23	1	0.547238	98.5520%			98.55%		
62	T5-37	4	0.549521	98.5460%					
63	T5-37	1	0.549726	98.5460%					
64	T5-37	3	0.549892	98.5450%					
65	T5-37	2	0.549988	98.5450%				98.55%	

RANKING TABLE FOR A1-s1mET4

RANK	CLASS	MEAN %	S.D. %	p-RATIO
1	A	99.6880%	0.00321	311
2	T9-41	99.3338%	2.05E-05	48470
3	T8-40	99.1783%	1.26E-05	78819
4	T11-43	99.1698%	0.00103	963
5	T10-42	99.1618%	0.001126	881
6	T7-39	99.0003%	6.6E-05	14996
7	B	98.8663%	0.001236	800
8	T12-44	98.8548%	0.00155	634
9	T6-38	98.7708%	0.001582	624
10	C	98.6725%	0.000308	3209
11	D	98.6233%	6.65E-05	15040
12	T1-21	98.6073%	0.000272	3629
13	T2-22	98.6045%	1.29E-05	76379
14	E	98.5720%	0.000107	9205
15	T4-24	98.5545%	1.73E-05	56900
16	T3-23	98.5543%	2.06E-05	47806
17	T5-37	98.5455%	5.77E-06	170685

CTRL	CLASSES	COLOUR
vCJD	A	
sCJD	B	
Neuro	C	
Healthy	D	
GRB norm	E	

Rank matching table matching a vCJD known control (single replicate of 4 replicate scans) to other known and test sample replicate scans from clinical groups A-D alongside a single control from group E.

The match ranking method still does not improve the ability to identify unknown samples with confidence. Test 22 (B3: sCJD) and Test 21 (A3: vCJD) are still located away from the other CJD samples towards the normal samples, and the neurological non-CJD samples are scattered across the mid range of the ranks. However many normals and CJD clinical samples still segregated at opposite ends of the range, which infers the presence of a unique combination of physiological factors which make these samples different.

A number of strategies, such as applying filters to the spectral data, image analysis and other various intuitive methods based on the experience of the Radar World team, were tried for analysis to identify unknown samples, but it became evident that the match ranking method was not sufficient to reliably discriminate all clinical samples. At this stage the identities of the remaining test samples were un-blinded so that the entire data set could be used as a training set to develop an expert system analysis for the identification of clinical and control samples.

8.4.3 Analysis of variance classification: Expert Systems Analysis.

An expert system was developed based upon analysis of variance of frequency data within groups and between groups. Studying firstly the similarities, and then the differences between different samples groups produces a series of relationships which can be used to develop logic relationships and an expert system. Radar World has used this method in various applications ranging from coastal surveys by satellite imaging to the discrimination between sound and rotten timbers by a customised ADR apparatus. The identification of robust sets of P-value relationships for each sample group allows the classification of test samples. The P-ratios of variance

between and within groups are calculated by rank and cluster analysis of ADR-ratio data using Radamatic software.

Four scans of the first training samples from each class (A1, B1, C1, D1, and E1) 20 % as a training set, were used to compute P-value tables producing a series of 13 parameters to define each sample class. Each parameter produced a series of defining statistics and relationships between sample classes. For example, for the first test variable the between group statistics difference between samples show that sample B was 3 times that of sample A, that sample B was 2.7 times that of sample C, and sample D was 2.1 times that of sample C. Similarly if we consider the third test variable statistics, sample A is 10619, sample B 68392, sample C 11198, and sample D 24207. The summation of these relationships between sample classes for all 13 indices led to the development of an expert system of analysis able to characterise different sample classes.

This left 14 unknown samples from classes A-E to be identified using this expert system (the second sample in each set was kept aside for later validation). This clearly identified with certainty test samples 22 and 39 as sCJD, and test samples 21 and 40 as vCJD, which is also fairly evident from the match ranking tables shown in section 8.4.2. All other unknown blind test samples from classes A-E were not identified with 100 % reliability but gave high P-values ranging from 93 – 99.9 % in probability, however this was not good enough for certainty and reliability. To improve this, values for those correctly identified samples were introduced into the P-value computation to produce revised parameters. Revision in these parameters enabled the correct classification of all blind samples with 100 % certainty and reliability. The second set of control samples from each class which were reserved

(A2, B2, C2, D2, E2) and were not used in any matching or P-value assessments were used to validate the knowledge base. They were correctly identified with 100 % confidence.

Due to the variability between patients and different blood types the identity of 20 % of all samples was necessary for training to ensure correct classification of unknown samples. Therefore if we have 100 unknown samples then 20 need to be revealed to correctly identify the remaining 80 at the 0.001 % significance level, or 0.05 % confidence level.

The results described so far shown that the match ranking method cannot distinguish the clinical classes of unknown samples with high certainty, but it does indicate that many vCJD and sCJD blood samples are sufficiently different from normal blood to segregate as a distinct group least like normal blood. The P-value expert system approach looks much more robust, and may yield a useable system after further evaluation. This would suggest that there are distinct physiological markers present in whole blood of patients with sCJD and vCJD that can be detected using ADR spectroscopy.

In the development of the P-ratio expert systems analysis described in section 8.4.3 samples were un-blinded to aid its development. Therefore to evaluate the suitability of the expert system for the identification of clinical CJD blood samples a second small blind study was performed.

8.5 Study design

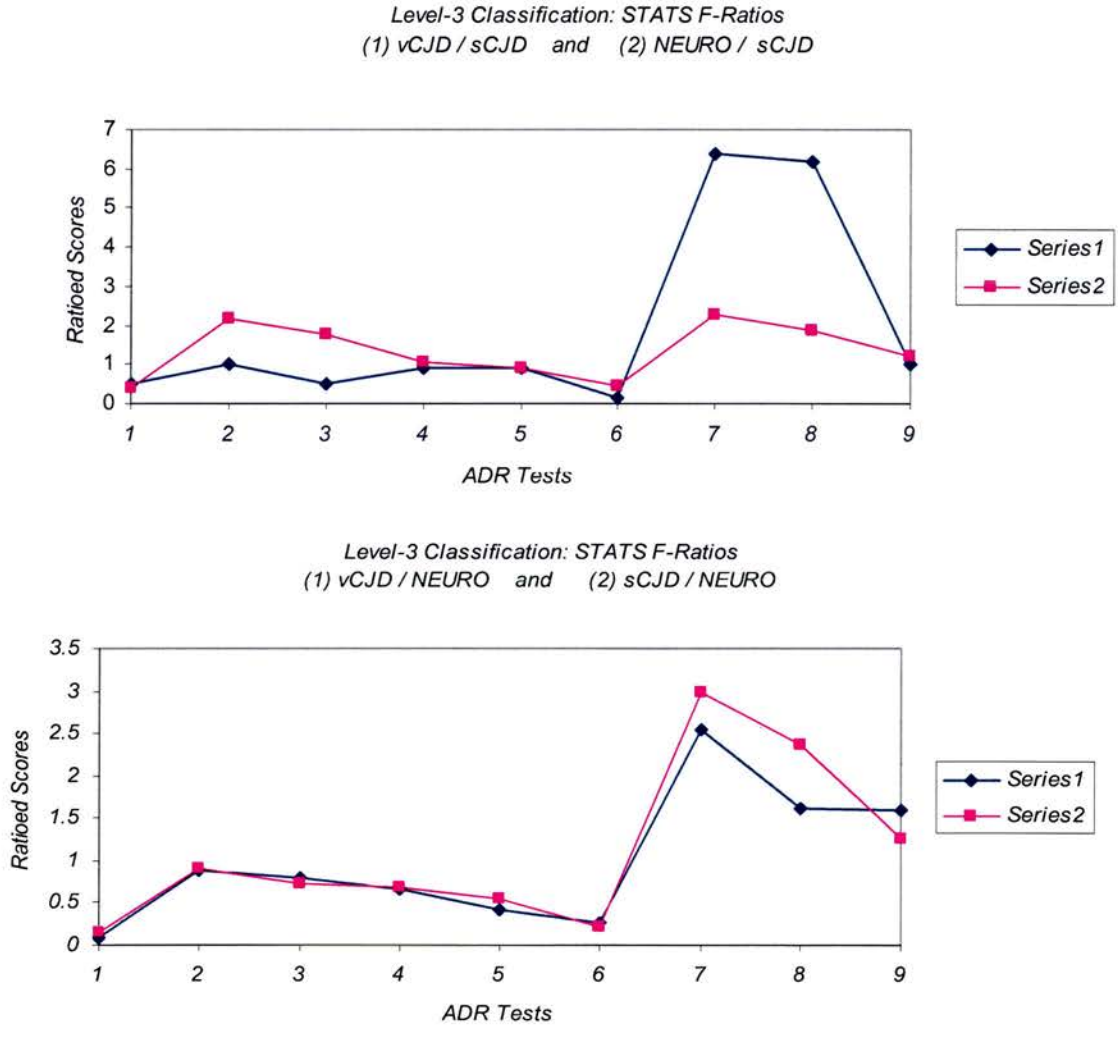
10 whole blood samples were obtained from the National CJD Surveillance Unit frozen archives. Samples included 4 vCJD, 3 sCJD, and 3 samples from non-CJD neurological controls. The controls were obtained from patients initially referred to the unit as possible CJD cases but which subsequently proved to be non-CJD. It was ensured that all samples had not been included in the first ADR spectroscopy study. 0.5 mL whole blood samples in 2mL storage tubes were vortexed and then allowed to settle for 10 minutes prior to ADR scanning. 4 scans of each sample were taken and scans of an empty storage tube was taken so that this background data could be subtracted from sample scans so that spectral data was for the blood sample alone.

8.5.1 Data analysis: variance classification

Analysis of the ADR spectra generated in this second blind study was performed as described in section 8.4.3. Frequency analysis of ADR spectral scans produced ADR-ratio data. Rank and cluster analysis using Radamatic software was used to produce P-ratios of variance which produced a range of indices used as discreet classifiers for samples groups. This is referred to as a 'proper analysis of variance'. The P-variance data classification parameters produced for vCJD, sCJD, and neurological sample groups in 8.4.3 were used to correctly identify the blind samples in this second study. For example, sample 7 was a whole blood sample from a sCJD patient and the percentage correlation of oscillation between it and 3 controls from vCJD, sCJD, and neurological control classes were used for supervised clustering. Its correlation was 0.981 with the sCJD control compared to 0.939 with the vCJD control and 0.948 with the neurological control. This sample was correctly classified

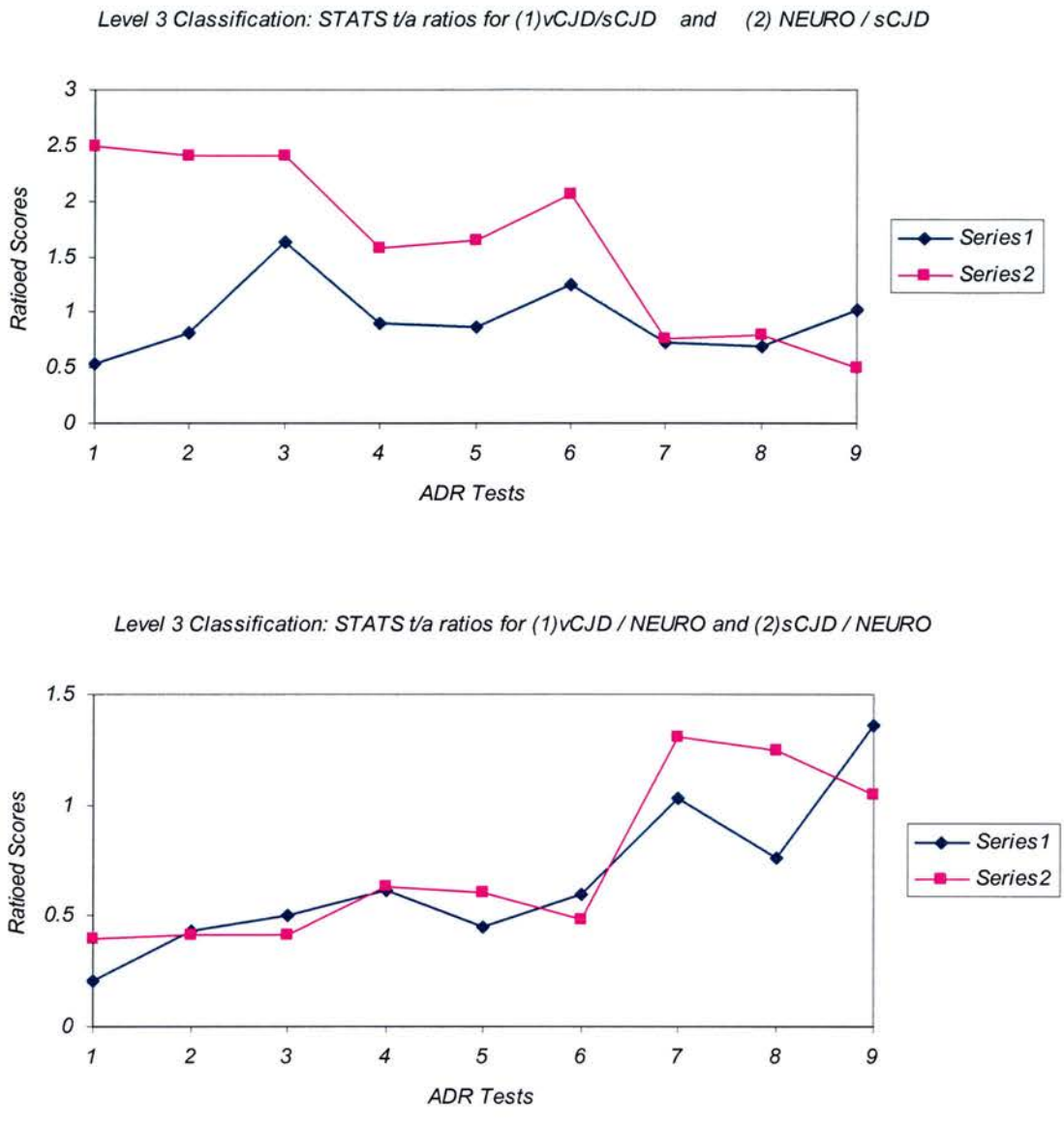
as sCJD. All other blind samples were correctly classified in the same way. To illustrate indices which were used to correctly classify samples ADR-ratio and time (t) / amplitude (a) plots for nine of the ADR test parameters were plotted for ratios of different clinical groups. These graphs are presented in Figures 59 and 60 below, the separation between sample series ratios illustrate how different ADR test indices can be used to distinguish a particular clinical group from another.

Figure 59



Ratio scores for series of ADR tests illustrate the separation of different sample class ratios

Figure 60



Ratio scores for series of ADR tests illustrate the separation of different sample class ratios

In this chapter we have presented proof of principle of a potential method employing ADR spectroscopy and an expert systems statistical spectral analysis for the classification of sCJD and vCJD clinical and neurological control and healthy adult whole blood samples. The developed classification system was then validated by a small blind study. The findings suggest that there are distinct physiological markers detectable in clinical whole blood samples from vCJD and sCJD patients by ADR spectroscopy. These methods present a rapid, non-destructive test system for the antemortem diagnosis of patients with CJD that is not dependent on the detection of PrP^{Sc} for their classification. The number of samples analysed in these studies is very small and a much larger validation study of healthy adult controls, clinical CJD, non-CJD control samples, along with samples from patients with a wide spectrum of neurological, infectious and non-infectious conditions is required to assess to what extent the expert system statistical parameters are specific for each group. Although the exact nature of the ADR spectral alterations is unknown it may be that they are due to distinct combinations of blood physiological features for particular disease classes rather than due to the detection of PrP^{Sc} as was the assumption with the mouse scrapie study. A panel of blood group typing samples was used to train the system to recognise differences associated with different blood types so to increase the reliability of the system analysis. This approach presents a unique diagnostic approach for the detection of CJD blood samples, and it is possible that with further study these methods may be adapted for the detection of other diseases.

Discussion

This thesis presents a detailed study of the expression of PrP in the peripheral blood of patients with vCJD and sCJD using DELFIA and flow cytometry techniques, and describes the development of DELFIA based assays for the detection of PrP^{Sc} in blood and other tissues. In addition ADR spectroscopic methods have been investigated as a potential means of distinguishing vCJD and sCJD blood samples from healthy adult and neurological control samples.

The DELFIA assay technique described in chapter 3 for the detection of PrP^{c} has been used previously [9] and is well suited to the screening of whole blood and separated components of blood from clinical and control samples for PrP^{c}. The assay is quick and has a high sensitivity for the detection of PrP^{c}. DELFIA technology is compatible with high throughput and has a high signal to background ratio, due to background deteriorating rapidly while the signal has a long lifetime. Flow cytometry analysis, which is also used in this chapter for an analysis of PrP, has been used previously to illustrate the expression of PrP on healthy adult human blood cells [6,10].

No previous studies have evaluated the concentration and expression of PrP in the whole blood and separated components of patients with vCJD. DELFIA analysis of separated components from controls and clinical patients found the highest levels of PrP^{c} associated with the platelet and plasma components and much lower levels associated with red blood cells, which is in agreement with previously described findings of Macgregor et al [9]. Levels of PrP^{c} in the leukocyte-enriched buffy coat

fractions were very low, often at the detection limit of the assay, and these levels could not be accurately calculated particularly in clinical groups, hence their exclusion from further analysis. The separation method used here for the preparation of separated blood components was a compromise for greatest utility of archived samples, and was not ideal for isolation of the platelet and buffy coat components; cells often clumped together and proved difficult to resuspend, this may explain the large median ranges in the platelet data, and the low levels of detectable PrP^c in buffy coats. These large ranges in platelet PrP^c concentrations are not supported by any large fluctuations in platelet counts as seen in haematological data for patients and controls used in this study; the data does not show any abnormalities.

In the analysis of whole blood there was a statistically significant decrease in the concentration of PrP^c in vCJD ($p=0.005$) and neurological control patient samples ($p=0.0001$) compared to healthy adults but not when compared with the sCJD group. There was significant elevation ($p=0.022$) in the plasma PrP^c concentration in sCJD patients when compared with both healthy adult and neurological control groups; however no significant differences were evident between levels in sCJD and vCJD patients. The elevation of plasma PrP^c in sCJD patients agrees with a previously published report; however in this study a distinct finding is that levels of plasma PrP^c in sCJD patients were significantly elevated by comparison with healthy and the neurological control group, which was not the case in previous published findings [164]. These differences may be a consequence of using neurological controls with conditions other than Alzheimer's disease, as was the case in the previous study. There was a significant reduction in the concentration of red cell PrP^c in neurological

controls compared with all other groups. Flow cytometry analysis also demonstrated significantly less PrP expression on red cells of neurological controls than healthy controls. These results would appear to indicate that there is less PrP^c per red blood cell in neurological controls compared with other groups. It is difficult to explain the reduced expression of PrP on red blood cells. It was considered that the differences between groups might be a consequence of the age of patients affected by vCJD, sCJD, and neurological disorders, however no relationship between age and PrP^c concentration was found in either control or clinical sample groups. It is possible that the reduced concentration of whole blood PrP^c in vCJD patient samples might be due to the conversion of PrP^c to PrP^{Sc} as part of the replication process involved in disease pathogenesis. The absence of a similar reduction of whole blood PrP^c in sCJD patients would be expected since PrP^{Sc} is largely confined to the CNS and replication of PrP^{Sc} in the periphery prior to neuroinvasion is not a feature of sCJD. Alternatively the vCJD whole blood reduction could be a summation of reductions in PrP^c in the plasma and platelet components, whereas in sCJD the low platelet PrP^c value is offset by the high plasma value so balancing out in whole blood levels. It is likely that the reduced levels of whole blood PrP^c in neurological controls is a consequence of the reduced PrP^c levels found in red cells and platelets in this group. The possibility was considered that increases in sCJD plasma PrP^c levels may be a consequence of its release from platelets, since platelets are known to express PrP^c on the cell surface and to store PrP^c in alpha granules which is released into plasma in a soluble form on platelet activation [150,165]. To support this theory there was a significant reduction in the platelet PrP^c concentration in sCJD patient samples compared to healthy adults implying that platelets may have shed PrP^c into plasma.

The expression of PrP on platelets of vCJD and sCJD as determined by flow cytometry was elevated compared with healthy adult and neurological controls, and although this elevation did not reach significance perhaps this represents upregulation of PrP prior to its release into plasma Investigations of cell surface PrP on platelets did not implicate blood sample storage and transit as a possible cause for increased cell surface PrP, it found cell surface PrP on platelets decreased with sample age.

There was no evidence of a reduction in the expression of PrP on lymphocytes of patients with sCJD when compared to neurological controls in contrast to a previous report [166]. However, here both 4F2 and 3F4 anti-PrP MAbs were used together for flow cytometry studies, whereas the previously reported study used 3F4 alone which does not to bind to human red cells [6]. It is possible that MAb 3F4 might show reduced binding to PrP^c on lymphocytes in some clinical conditions if PrP^c expression were altered or PrP^c interacted with other membrane components due to lymphocyte activation [167,168]. PrP on red cells, platelets and lymphocytes was reduced to background levels after PK treatment in all clinical and control groups, indicating the absence of any detectable cellular expression of abnormal disease associated forms of PrP.

These findings indicate that a reduction in concentration of whole blood PrP^c may be common in vCJD and other neurological diseases but not sCJD. An elevated level of plasma PrP^c may be common in sCJD. These differences between groups appear genuine, presumably reflecting differences in the disease process in the patients in these particular groups and not an artefact of age, specimen collection, storage or

analysis. Despite the significance of differences between groups the variation in values are large and there is considerable overlap between CJD groups and control groups, which rules out the exploitation of these differences in whole blood and plasma in screening strategies. These studies expose the limitations in the use of blood PrP^c levels as a diagnostic tool. However they illustrate important observations on the distribution of PrP^c in the peripheral blood of CJD patients and the potential of DELFIA-based PrP assays in clinical practice. The analytical sensitivity of DELFIA-based assays used here represent a significant step towards the development of DELFIA for the detection of PrP^{Sc}, which is a much more reliable indicator of infection, and the findings emphasize the need to develop assays for its detection in blood [21,152].

Use of PK and GdnHCL in the Development of assays for PrP^{Sc}

The development of a sensitive DELFIA based assay for PrP^{Sc} was the main focus of the remainder of this thesis. Part of the initial development process involved an assessment of the suitability of using existing methods employing PK and GdnHCL to discriminate between PrP^c and PrP^{Sc}. The partial resistance of PrP^{Sc} to proteolysis by PK was first described by Prusiner et al [2] and remains a key means of discriminating between PrP^c and PrP^{Sc} in existing western blotting, immunohistochemical and ELISA based assays for post-mortem TSE diagnosis. Studies into the susceptibility of untreated healthy adult human whole blood PrP^c to proteolysis with increasing concentrations of PK found that PK could be used successfully to eradicate almost all PrP^c in samples. These studies however highlighted one of the major problems of using PK as a means of removing PrP^c, it

has a broad specificity and since PrP^c levels are likely to fluctuate between samples its optimisation would require constant readjustment to ensure that conditions of proteolysis were sufficient to remove PrP^c but not PrP^{Sc}. If PK concentrations are too high they may remove PrP^{Sc} as well as PrP^c because the resistance of PrP^{Sc} to PK is not absolute, and if PK concentrations are too low they will not remove all PrP^c [2]. The use of PK will therefore always be technically difficult and limit sensitivity. Also, PK appeared to degrade the capture monoclonal antibody FH11 affecting antigen capture and therefore the mechanics of binding and accurate detection of PrP^c. This was shown in preincubation studies described in section 4.3.

As an alternative to PK, GdnHCL was assessed as a means of distinguishing PrP^c from PrP^{Sc}. The use of GdnHCL in these assays has been described previously by several groups [21,152,158]. The methods of Barnard et al [158] for the differential extraction and separation of PrP^c from PrP^{Sc} in BSE infected bovine brain tissue were well suited to the isolation of PrP^c from healthy adult whole blood samples. This methodology was also used successfully to distinguish 263K-scrapie infected hamster brain homogenate from uninfected healthy hamster controls, and to detect PrP^{Sc} in a vCJD brain homogenate spiked plasma log dilution series. The CDI assay described by Safar et al [21] was adapted successfully for the detection of human PrP^{Sc} in vCJD spiked human plasma samples. A comparison of candidate monoclonal capture antibodies found that antibody 1120-64-09 was best suited to this type of assay because it did not show different binding affinities for native and denatured PrP as was the case with the capture antibodies FH11 and D18. MAb 1120-64-09

had a superior signal for the detection of plasma PrP^{c} and PrP^{Sc} purified from vCJD brain material.

Development of the CDI assay for PrP^{Sc} detection

The CDI assay employing monoclonal antibody 1120-64-09 as capture antibody and europium labelled MAb 3F4 as detection antibody was selected for further development. NaPTA and NaCl precipitation techniques described previously [21 160] were adapted and used successfully to precipitate and concentrate PrP^{Sc} from larger volumes of vCJD brain spiked human plasma prior to detection using the CDI assay. These precipitation methods may permit the detection of PrP^{Sc} in blood or tissues where it is present at very low concentrations because they effectively concentrate PrP^{Sc} from larger quantities of sample. Claims by Safar et al [21] that NaPTA binds specifically to oligomers and polymers of PrP^{Sc} but not PrP^{c} were not supported by experiments presented here. PK treatment before NaPTA precipitation and CDI analysis increased the sensitivity for the detection of PrP^{Sc}. In addition a traditional salting out protocol using NaCl, adapted from Polymenidou et al [160] had a very similar sensitivity to that of NaPTA precipitation for the detection of PrP^{Sc}. These experiments do not support a specificity of NaPTA for PrP^{Sc} alone, unless proteolysis of PrP^{Sc} increases the affinity of the binding interaction between it and the capture antibody. The increases in sensitivity are most likely caused by the removal of PrP^{c} which competes for binding to the capture antibody, and, or other proteins which compete non-specifically. Despite this the CDI assay and NaPTA precipitation methods were adopted to develop a sensitive assay for the detection of human PrP^{Sc}. The assay has a maximum limit of detection of 10^{-5} log dilution for 10 % vCJD brain homogenate spiked into human control platelet poor plasma. This represents the

most sensitive reported assay in existence for the detection of PrP^{Sc} when compared with other promising assays (see Table 5 pp.62). The developed NaPTA precipitation and CDI assay techniques were used to detect PrP^{Sc} in spleen, tonsil, and muscle tissues from patients with vCJD, sCJD, and non-CJD neurological controls. PrP^{Sc} was detectable in spleen and tonsil homogenates from patients with vCJD. PrP^{Sc} was not detected in sCJD tonsil samples but was detectable in a single sCJD muscle sample. These studies verify the suitability of this assay for the sensitive detection of PrP^{Sc} in peripheral tissues. Any physiological differences or structural abnormalities that may exist between the type of PrP^{Sc} in brain and in peripheral tissues do not restrict its detection using these methods. The sensitivity of this method shows promise for the detection of PrP^{Sc} in the peripheral blood of patients with vCJD and is certainly a candidate assay for consideration as a means to ensure the safety of blood and blood products used in the transfusion services.

ADR spectroscopy study

Finally ADR spectroscopy techniques were employed in an analysis of sCJD and vCJD clinical and neurological control, healthy adult, and spiked whole blood samples. Spectral analysis, rank matching, and expert systems statistical analysis methods were explored to evaluate methods able to correctly identify blinded clinical and control samples. Expert systems statistical analysis approach was found with training to be best suited to the identification of blind samples with 100 % certainty and reliability. A second small validation study of clinical CJD and control whole blood samples presented proof of principle of employing ADR spectroscopy and expert systems statistical spectral analysis for accurate classification.

Recent developments and conclusion

In 1997 the Transfusion Medicine Epidemiology Review (TMER) study funded by the Department of Health was set up to study potential links between vCJD and blood transfusion. The first case of possible transmission of vCJD by blood transfusion was identified by this study was reported in early 2004 [76], and is described in section 1.6.5. The report identified a further 17 individuals who had received blood products from donors who subsequently developed vCJD. During the writing of this thesis there have been further critical developments concerning blood-borne iatrogenic vCJD transmission. A report in the Lancet in August 2004 described a preclinical case of vCJD infection identified by autopsy which appeared to have been transmitted by blood transfusion [169]. The case identified was one of the 17 individuals who had received blood products from donors who subsequently developed vCJD. The patient was elderly and had received a donation of non-leucodepleted red blood cells in 1999 from a donor who developed vCJD 18 months after donation. The donor died in 2001 and vCJD was confirmed after autopsy. The recipient died in 2004, 5 years after receiving the transfusion with no signs of a neurological disorder. It is interesting to note that in both reported cases of transfusion-related transmission of vCJD the donor received non-leucodepleted red blood cells products which are relatively unprocessed compared with fractionated plasma products which are subject to processing steps which remove 3 to 5 logs of infectivity [131]. A medicolegal instruction for autopsy was issued and allowed detailed examination of the patient. The patient was heterozygous for Methionine / Valine at codon 129 of the *PRNP* gene. PrP^{Sc} was detected by western blot in the spleen and cervical lymph node but not in the brain. This case represents the first

recorded case of autopsy detected pre-clinical vCJD infection in the UK. The identification of this case is critical for a number of reasons. This is the first known case of vCJD, be it sub-clinical, detected in an individual who is heterozygous at codon 129 of the *PRNP* gene. To date all clinical reported cases of vCJD have been homozygous for Methionine at this codon, and considering that the heterozygous genotype represents the largest genetic subgroup in the population this case has important implications for future predictions of the number of vCJD cases considering that there has been uncertainty as to the susceptibility of this genetic sub-group. One possibility is that after exposure to the vCJD infectious agent heterozygous individuals have a longer incubation period than homozygotes with infection remaining at sub-, or pre-clinical levels for many years. This is particularly critical since such individuals may represent a significant source of iatrogenic transmission either via blood transfusion or the contamination of surgical instruments by lymphoreticular tissues. If sub-clinical vCJD infection in this genotype is prevalent it at present remains undetected and uncontrolled. In the absence of a screening test for vCJD these individuals could be a major source of future iatrogenic transmissions. Indeed there is the potential for iatrogenic vCJD transmission cases to exceed those caused by BSE. The possibility remains that both apparent transfusion-related transmission cases may be a consequence of exposure to BSE through contaminated beef, however statistically the chances of observing vCJD transmission in two recipients of blood donated from patients who subsequently died of vCJD will be considerably less than the 1/15000 to 1/30000 odds reported for the first case [76].

This most recent case of transmission by transfusion highlights the increasing need for preclinical blood screening tests for vCJD to enhance the safety of blood transfusion, to reduce future iatrogenic transmission of vCJD between individuals by identifying those individuals harbouring sub-clinical infection. The need for such a test is compounded by recent studies by Gregori et al [170] which in an assessment of leucodepletion filtering found that it removed only 42 % of total TSE infectivity in 263K-scrapie endogenously infected hamster blood. Universal leucodepletion was introduced by the UK blood transfusion services in 1998 as a response to advice that infectivity if present was mainly associated with the buffy coat and plasma. This was closely followed by the decision in October 1999 to import all plasma for fractionation used to manufacture plasma derivatives from the United States.

In this thesis a CDI assay incorporating NaPTA precipitation and PK treatment has been developed with a sensitivity of 10^{-5} for the detection of 10 % vCJD brain homogenate, and $10^{-2.5}$ for 10 % vCJD spleen homogenate. NIBSC spleen and brain preparations were used as a source of PrP^{Sc}. This represents the sensitive detection of 10^{-6} and $10^{-3.5}$ for unhomogenised brain and spleen tissue respectively. At present this assay represents one of the most promising PrP^{Sc} assays with a sensitivity approaching levels at which it might be possible to detect PrP^{Sc} in blood. At present the NIBSC working reference preparations of vCJD brain and spleen homogenate are undergoing assessment for infectivity in the hope that levels of infectivity can be equated with levels of PrP^{Sc}. Accurate infectivity titres were hoped to be obtained from chimeric mouse-human transgene expressing mice but these were insensitive to infection with vCJD but readily infected with sCJD [171]. Titres in wild type mice inoculated intracerebrally are approximately 1,000,000 infectious units (IU) per gram

for vCJD brain and 1000 IU per gram for vCJD spleen. Whether this represents infectivity titres for humans considering the species barrier and route of infection is open to question. Studies in rodent models [172] predict that infectivity in human blood will be in the region of 10 IU per mL. Therefore a PrP^{Sc} assay able to detect levels associated with < 1-10 IU / mL of blood may be of use for screening. Titres of PrP^{Sc} in vCJD brain and spleen homogenates in this thesis have shown that differences in the levels of PrP^{Sc} reflect the same reported levels of difference in infectivity between these tissues, and given recent reports by Legname et al [173] that PrP^{Sc} is in fact the infectious agent, it may be at last be possible to refer to PrP^{Sc} as the infectious agent. If PrP^{Sc} levels are synonymous with infectivity then the sensitivity of the CDI assay assessed with NIBSC vCJD brain and spleen homogenates presented here would equate to a limit of detection of 1 IU which is a level of sensitivity perhaps capable of detecting infected blood. It may not be quite so straightforward; the assay may not be suited to the detection of PrP^{Sc} in human peripheral blood. PrP^{Sc} here may be less aggregated or in a different form than that in spleen and brain tissue, and may be more susceptible to proteolysis with PK used to achieve the sensitivity in brain and spleen assays. Although results presented in this thesis do not suggest that differences which might exist between vCJD brain and peripheral tissues in terms of patterns of glycosylation, protease sensitivity, and epitope presentation affect the ability of the CDI assay to detect PrP^{Sc}, this may not be the case with blood. The testing of clinical vCJD blood samples may be useful to ensure that this assay approach is able to detect PrP^{Sc} in blood but such experiments would not validate the capability of the assay to detect infectivity in the asymptomatic phase. This represents a significant problem; one of few alternatives

is to assess assay sensitivity using blood of known infectivity from experimentally or natural TSE infected animals. It is probable that the use of animal TSE blood will constitute all validation studies. However in the case of the CDI assay described and assessed in this thesis the specificity of monoclonal antibodies would not be appropriate for the detection of ovine or murine PrP. Substitution with antibodies of the required specificity may not afford the same sensitivity of detection. In addition the form of PrP^{Sc} in animal blood may not be the same as that associated with human blood. In the absence of preclinical blood samples from individuals known to be incubating vCJD, the validation of promising assays using clinical vCJD blood samples and blood from experimentally or naturally occurring animal TSEs remains the only alternative. Therefore if the CDI assay is to be considered as a candidate assay for blood screening, this would be the next step in its validation. The issue of specificity is also critical for all screening tests when the ethical considerations of a positive test and its adverse effects upon blood supply with the removal of false positive donations and the negative impact upon donor recruitment, is considered. A specificity of 100 % would naturally be desirable. In a recent publication by MacGregor at al [174] a false positive rate of greater than 1/200 or specificity of 99.5 % was deemed unacceptable. Considering that in the UK there are 2.5 million donations per anum, a screening assay with 99.5 % specificity would yield 12500 false positive donors and this would have a significant impact upon the blood supply chain. Specificity is therefore of paramount importance. Sensitivity is less critical since the identification of some if not all infected blood donors would significantly contribute to curtailing the risk of iatrogenic transmission of vCJD by blood transfusion despite not detecting all positive donations. A confirmatory assay would

be essential, reliant upon a different approach to retest those positive blood samples identified using the first screening assay, probably an assay based on the detection of infectivity. It would require a sensitivity similar to the first assay in the region of 1-10 IU / mL. One possibility would be to use a mouse bioassay although this would take considerable time to produce results. In-vitro assays such as one producing results in 2 weeks reported for a scrapie strain by Weissmann et al [175], or the knock-in mouse expressing humanized chimeric PrP spleen bioassay able to produce results in 30 days described by Kitamoto et al [176] would be ideal as confirmatory assays for human blood screening. Perhaps with further validation the ADR-spectroscopic analysis technique presented in this thesis may be of use as a screening assay with the CDI assay used as a confirmatory assay for the detection of PrP^{Sc}. Whether assays used are for infectivity, PrP^{Sc} or surrogate markers of vCJD infection they would need to be quick, cheap, and suitable for the screening of all UK donors.

The ethical considerations of introducing a blood screening test are a major issue for the UK blood transfusion services [177]. The transfusion services are under increasing pressure to introduce a vCJD-screening test once a viable option becomes available, and this pressure has been compounded by the two recent reports of transfusion-related transmission. In the past the policy of the UK transfusion services has been to inform donors of the types of tests carried out on their donation and to contact and counsel those who produce a positive test. Screening tests are where ever possible implemented along with a confirmatory test to assure that test results are correct. In the case of vCJD there are many ethical difficulties with implementing a test without being certain of the implications of a positive test result, whether this does in fact

equate to an increased risk of vCJD, and in the absence of any tried and tested therapeutic strategies. Indeed these ethical issues are being faced presently as a consequence of the decision of the CJD clinical incidents panel to inform all recipients of blood components donated by individuals who later developed vCJD. These individuals were informed of the risk. They were advised that they could no longer donate blood and also that surgical precautions would be required. However there is considerable uncertainty as to whether these individuals will actually develop vCJD; and more significantly there are at present no available tests to investigate the risk. Despite these ethical considerations it is essential that any available blood-donor screening test be implemented to enhance the safety of blood transfusion and to reduce the spread of vCJD amongst recipients of blood products.

Much like vCJD screening tests, therapeutic strategies are at an early stage of development. Many show promise and could be useful for prophylaxis in preclinical vCJD patients. The use of bis-quinacrine is undergoing evaluation, and pentosan polysulphate, which showed promise in animal TSE infectivity models has been administered to vCJD patients on an ad hoc basis. Quinacrine is currently being used in the Prion-1 trials conducted by the MRC Prion Unit, London, UK. In addition the use of anti-prion antibodies and peptides, and beta-sheet breakers are alternatives also under development. Any effective treatment would be of most use at the preclinical disease stage since the neural degeneration associated with clinical disease is probably irreversible. Prophylactic treatments could be made available to blood donors and other individuals who produced a positive result with any implemented vCJD screening test so offsetting some of the ethical difficulties associated with implementing a screening test.

In the next 5 years it is likely that the most promising diagnostics for the detection of vCJD in blood will undergo necessary validation using vCJD clinical blood samples and preclinical blood samples from both natural and experimental TSEs in animals. It seems likely that NIBSC along with the UK blood transfusion services and the Department of Health will work closely together to evaluate and implement a screening assay to enhance the safety of blood transfusion and reduce the iatrogenic transmission of vCJD via transfusion. Therapeutic strategies will also be developed to produce prophylactics. Blood collection bag filter technologies are being developed and evaluated by both Pall Corporation and by MacoPharma in alliance with Pathogen Removal and Diagnostic Technologies Inc (PRDT). Pall reported in November 2004 at the annual meeting of the American association of blood banking that their Leukotrap® affinity prion reduction filter reduced infectious vCJD prions from red blood cell concentrates below the limit of detection of the western blot assay. The filtering technology had been shown previously to remove scrapie infectivity from scrapie infected mouse blood. The filter is a leucodepletion device which also removes infectious prions. The MacoPharma PRDT alliance has developed blood bags with ligands that absorb infectious prions from blood and blood derived products. Both companies are aiming to launch products in Europe in 2005, and it seems likely that these technologies will be implemented before a screening test. It seems likely that the blood transfusion services will implement further restrictions on donors and blood product manufacture and use. For example a recent ban on donors who received a blood transfusion after January 1980 was implemented in April 2004 and resulted in the loss of 3.2 % of blood donors, and options are being considered to reduce the plasma content of red blood cell

components. In addition a decision was taken in April 2004 to import fresh frozen plasma from the United States for all patients born after January 1996. The number of countries deemed to be free of vCJD from which blood and blood products can be sourced will probably fall, and the number of countries trying to import such products will rise, as other countries develop significant clusters of vCJD like the UK. This will mean a worldwide shortage of blood and blood-products. Also, while some countries are free of vCJD, there are other endemic risks to such blood which need consideration.

vCJD is clearly having a significant effect upon the blood transfusion services, and a screening test in place of such precautionary measures remains a priority. The CDI assay and ADR-spectroscopic techniques presented in this thesis represent two of the most promising techniques which require further investigation for their potential as vCJD blood screening tests. Further work is required to assess their ability to identify PrP^{Sc} and, in the case of ADR-spectroscopic methods, physiological markers for vCJD which would enable the identification of individuals incubating vCJD
It is likely that there will be further cases of vCJD transmission by blood transfusion, though without a highly specific and sensitive screening test it may not be possible to make an accurate assessment of the numbers of individuals pre-clinically incubating vCJD, and so it is difficult at this stage to predict whether or not iatrogenic transmission will lead to a much wider incidence of vCJD.

Appendix

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Variation in concentration of prion protein in the peripheral blood of patients with variant and sporadic Creutzfeldt-Jakob disease detected by dissociation enhanced lanthanide fluoroimmunoassay and flow cytometry

Tim Fagge, G. Robin Barclay, Ian MacGregor, Mark Head, James Ironside, and Marc Turner

BACKGROUND: A highly sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) and flow cytometry techniques have previously been developed and employed to characterize soluble cellular prion protein (PrP^c) expression in whole blood and separated components from healthy adult blood donors. No previous studies with these techniques have evaluated the concentration and expression of PrP in the blood of patients with variant Creutzfeldt-Jakob disease (vCJD).

STUDY DESIGN AND METHODS: For blood from vCJD patients, sporadic CJD (sCJD) patients, non-CJD neurological controls, and healthy adults, PrP^c was measured by DELFIA and cell-associated PrP was measured by flow cytometry.

RESULTS: DELFIA analysis identified a significant reduction in the concentration of PrP^c in the whole blood of vCJD ($p = 0.012$) and non-CJD neurological patients ($p = 0.0004$) compared with healthy adults. A significant elevation was found in plasma PrP^c in sCJD patients compared with healthy adult ($p = 0.022$) and neurological controls ($p = 0.050$). Flow cytometry found no significant differences between groups in expression of PrP on platelets and lymphocytes, nor in sensitivity of cellular PrP to proteinase K. Neurological controls show significantly less PrP on red cells than healthy adults.

CONCLUSION: There are differences in free and cell-associated PrP found in blood of CJD patients and control groups, some of which might be useful with other tests in disease profiling as an aid to diagnoses.

The human prion diseases or transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative disorders believed to be caused by a posttranscriptional conformational change in cellular prion protein (PrP) from its soluble form (PrP^c) to pathogenic protease resistant isoform PrP^{Sc}.[1] The most common of these is sporadic Creutzfeldt-Jakob disease (sCJD) but recently a variant form of CJD (vCJD) was identified in the United Kingdom[2] and has been linked to human infection by the bovine spongiform encephalopathy (BSE) agent. The presence of PrP^{Sc} in the peripheral tissues of patients with vCJD[3,4] and recent experimental transmissions of BSE and natural scrapie between sheep by blood transfusion raise the possibility of the potential for iatrogenic transmission in humans by

ABBREVIATIONS: BSE = bovine spongiform encephalopathy; DELFIA = dissociation-enhanced lanthanide fluoroimmunoassay; pK = proteinase K; PrP = prion protein; PrP^c = soluble cellular prion protein; PrP^{Sc} = pathogenic protease resistant prion protein; sCJD = sporadic Creutzfeldt-Jakob disease; TSE(s) = transmissible spongiform encephalopathy(-ies); vCJD = variant Creutzfeldt-Jakob disease.

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blood transfusion.[5,6] This risk has been highlighted by two recent reports of possible transmission of vCJD by blood transfusion,[7,8] which supports the likely presence of infectivity in the blood of preclinical vCJD patients. There is therefore a pressing need for preclinical screening tests, which either identify PrP^{Sc} or are able to identify infected individuals via the detection of reliable surrogate markers to enhance the safety of the blood supply.

The association of infectivity with blood is poorly understood, although there is growing evidence from studies in mice and hamsters that the initial TSE infection is carried by white blood cells (WBCs)[9,10] to lymphoreticular tissues before neuroinvasion of the central nervous system via the sympathetic nervous system.[11-16] Recent reports of the transmission of natural scrapie and experimental BSE between sheep by whole-blood and buffy-coat transfusion support the theory that infectivity is associated with, but not restricted to, the WBC component.[17] Given that levels of detectable PrP^{Sc} and infectivity in peripheral lymphoreticular tissues such as spleen and tonsil in patients with vCJD are 2 to 3 logs lower than levels detected in the central nervous system,[4,18] it is likely that PrP^{Sc} is present at extremely low concentrations in peripheral blood. Attempts to detect PrP^{Sc} in human buffy coat by Western blot have thus far proven unsuccessful.[4] Intracerebral inoculation of human buffy coat into susceptible mouse models has failed to demonstrate infectivity,[19] although this may be a reflection of small numbers of animals used as well as insufficient assay sensitivity.

Tests designed for the detection of PrP^{Sc} in blood would require a high level of sensitivity, probably several logs greater than those already in place for postmortem TSE disease confirmation in humans and slaughterhouse cattle testing. Brown and colleagues,[20,21] basing calculations on studies in rodent TSE models, hypothesized that at most PrP^{Sc} would be present in blood at a level of 100 infectious units per mL of buffy coat, equivalent to 1 to 10 pg per mL; additional studies predict 10 infectious units per mL of peripheral blood,[22] assuming that the ratio of infectivity to PrP^{Sc} found in rodent TSE models is similar to that in vCJD infected humans. Considering these difficulties in detection sensitivity, alternatives to PrP^{Sc} as surrogate markers for TSEs have been explored in the hope that they will provide a test able to distinguish healthy donors from those harboring preclinical TSE infectivity. Dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) technology was employed here to study potential variation in the expression of the normal prion protein isoform (PrP^{c}) in vCJD and sCJD in humans. This technology has been previously employed by us in the detection of prion protein in different blood components of healthy blood donors[23] when we found that two-thirds of all PrP^{c} was associated with the plasma component, one-fourth in platelets (PLTs), and the remainder associated with mononuclear WBCs and red cells (RBCs). We also showed that PrP^{c} is released from PLTs into plasma.[24] A similar approach has also been used in a report of an increase in PrP^{c} expression in plasma of patients with sCJD and neurological controls when compared with healthy blood donor controls.[25] The expression of PrP in the peripheral blood of patients with vCJD has not been previously studied. In this study, we report levels of PrP^{c} in whole blood and separated blood components of vCJD patients, sCJD patients, healthy adult controls, and neurological controls as measured with DELFIA. In addition, flow cytometric analysis of cell surface PrP expression on different blood cell types was carried out on fresh whole-blood samples from the same clinical and control groups.

MATERIALS AND METHODS

Collection of blood samples

Anonymized whole-blood samples from 30 healthy adult apheresis PLT donors were collected by the Scottish National Blood Transfusion Service Edinburgh and stored for 24 hours at 4°C before separation to mimic the conditions of collection of samples from CJD patients transported to the National CJD Surveillance Unit from around the country. Blood from CJD patients and neurological controls was left over from samples obtained by the National CJD Surveillance Unit for genetic analysis. Samples were not obtained solely for this study owing to the difficulties of obtaining blood from these patients. Whole-blood samples from 10 vCJD patients, 10 sCJD patients, and 8 neurological controls were used for DELFIA studies. Informed consent was obtained from patients and donors for experimentation and ethics approval for the study was obtained from the Scottish Multi-Center Research Ethics Committee. All vCJD and sCJD cases had a probable or definitive diagnosis based on internationally established criteria.[26,27] Neurological controls were samples referred to the CJD Surveillance Unit from patients who subsequently did not meet criteria for a diagnosis of definite or probable CJD. These 8 patients were subsequently diagnosed with neurological disorders distinct from CJD including Alzheimer's disease (2), paraneoplastic syndrome (2), mitochondrial disease (1), Lewy body dementia (1), nonorganic depression (1), and central pontine myelinolysis (1).

All whole-blood samples were handled, separated, and stored in the same manner to ensure groups were directly comparable in scientific investigations. Whole-blood samples were collected into 9-mL vacuettes containing 1 mL of 3.2 percent trisodium citrate (Greiner Bio-One Ltd, Gloucestershire, UK). Blood for flow cytometry (0.4 mL) was set aside fresh as samples arrived at the National CJD Surveillance Unit and tested immediately. Samples were then separated into whole-blood, PLT-poor plasma, RBC, PLT, and buffy-coat components for

archiving frozen for these and other studies. The separation protocol was designed to produce these components without the need to use Ficoll. Ficoll, like dextran sulfate, is a polyanion, and it was thought that it may interfere with the processing and replication of the infectious agent as has been reported for dextran sulfate.[28] A quantity of 1.5 mL of whole blood was transferred to a sterile 2-mL tube, and the remaining volume was centrifuged at 450 × *g* for 10 minutes in a centrifuge (Model 4-15C, Sigma Aldrich, UK).

The PLT-rich plasma was removed and centrifuged at 16,060 × *g* for 10 minutes in a centrifuge (Heraeus Biofuge, Kendro Laboratory Products, Sollentum, Germany); the resulting PLT-poor plasma supernatant was transferred to 2-mL storage tubes. The buffy coat and the RBCs were transferred into separate 15-mL tubes, and 10 mL of phosphate-buffered saline (PBS; Sigma Aldrich, P4417) was added before the tubes were spun at 180 × *g* for 10 minutes. The supernatant and the top 0.5 mL of the interface from the RBC tube were discarded, PBS was added to the 14-mL mark, the tube was centrifuged at 180 × *g* for 10 minutes, and the supernatant was discarded. The pellet was resuspended in PBS to double the volume giving a 50 percent solution of cells, which were transferred to 2-mL storage tubes. The supernatant from the buffy-coat tube was centrifuged at 16,060 × *g* for 5 minutes to pellet the PLTs. The supernatants were discarded and the pellets were washed with PBS, resuspended in 1 mL of PBS, and transferred to a 2-mL storage tube. The buffy-coat pellet was washed in 14 mL of PBS, the supernatant was discarded, and 13 mL of distilled water added to shock-lyse the RBCs. One milliliter of 10× PBS was added before centrifugation at 180 × *g* for 10 minutes. The supernatant was discarded and the pellet was washed with PBS and centrifuged at 100 × *g* for 10 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of PBS. All separated components were stored at −80°C. Protease inhibitors were not used in the preparation of these components.

DELFIA

The highly sensitive method of time-resolved DELFIA was employed for the detection of PrP^c by sandwich immunoassay with methods described previously by our group.[23] The assay was calibrated with a dilution series of an expired PLT concentrate as a source of human PrP^c. This had been calibrated as a standard with recombinant human soluble PrP (Prionics, Zurich, Switzerland); 1 unit was found to be equivalent to 26 pg per mL. In addition a quality control plasma sample was included to confirm assay reproducibility. Blood component aliquots were thawed and assayed at five dilutions in duplicate. Suitable dilutions for each component were established in earlier experiments (data not shown) to ensure parallelism between dilutions. The means, median, and standard deviation were calculated and the U test was used to determine the significance of differences between groups.

Total protein measurement

Data were expressed in units per milligram of total protein for whole blood and all separated components to normalize data by compensating for the effects of the separation protocol upon cellular particulate recovery in components and to ensure that range differences in cell number did not contribute to differences in PrP^c levels between groups. Although studies on full blood counts of clinical patient samples and controls did not show abnormalities, full blood count data could not be generated from frozen stored clinical and control samples used in this study; therefore, normalization by total protein was essential for all components with the exception of whole blood.

Measurements of total protein levels were carried out with a protein assay (Bio-Rad, Hemel Hempstead, UK). The microtiter plate format was performed following the manufacturers instructions.

Flow cytometry

Blood samples. Samples were processed by flow cytometry immediately on arrival at the National CJD Surveillance Unit and subsequently assigned to clinical groups when diagnoses were made. An aliquot (0.4 mL) of whole blood from each sample was made available for flow cytometry studies. Any samples that did not fall into the diagnostic categories were not included in the analysis of results. Blood samples were also obtained from healthy adults described above and, because clinical samples were at least 24 hours old before arriving in the laboratory, these healthy adult control samples were processed by flow cytometry on the day following sampling.

Treatment with proteinase K. A quantity of 0.2 mL of the sample was set aside for staining (untreated) and the remaining 0.2 mL was washed once in 2.5 mL of cold cell wash (Becton Dickinson, Franklin Lakes, NJ), centrifuged down, and resuspended in 0.5 mL of proteinase K (pK) (Sigma Aldrich) at 1 mg per mL in Hanks' balanced salt solution with calcium and magnesium (Sigma Aldrich) and left at room temperature for 30 minutes (the pK concentration for complete removal of cellular PrP from healthy human blood cells under these conditions was determined by preliminary titration). The pK-treated sample was washed four times in 2.5 mL of cold cell wash, and the resultant cell pellet was divided equally between two 12 × 75-mm Falcon tubes (Becton Dickinson) for flow cytometry staining.

Flow cytometry staining and analysis. This was carried out essentially as previously described[29,30] except that a combination of monoclonal antibodies (MoAbs)

3F4 and 4F2 was used on the hypothesis that although MoAb 4F2 clearly identifies PrP on human RBCs whereas MoAb 3F4 does not bind to human RBCs,[28] the 4F2 epitope on any PrP^{Sc} may be more susceptible to pK cleavage than the 3F4 epitope. These MoAbs (5 µL each at 0.5 mg/mL) were added simultaneously to 100 µL of whole blood or pK-treated washed blood with 100 µL of cell wash. Second samples of whole blood and pK-treated blood received 100 µL of cell wash without anti-prion MoAbs as unstained (negative) control. After incubation and washing, anti-mouse immunoglobulin (human absorbed fluorescein isothiocyanate [FITC]-conjugated goat anti-mouse immunoglobulin $F(ab')_2$, Biosource, Camarillo, CA) at 1 in 500 in cell wash (100 µL) was added to all tubes. After incubation and washing, a sample (10 µL) was removed from each tube for RBC studies, and 5 µL of peridinin chlorophyll protein–conjugated anti-CD45 MoAb and 5 µL of phycoerythrin (PE)-conjugated anti-CD41 MoAb (Becton Dickinson) were added to each whole-blood tube. To each RBC study sample, 5 µL of PE-conjugated anti-glycophorin A MoAb (Serotec, Oxford, UK) was added. Samples were incubated an additional 30 minutes in the dark at room temperature. The RBC samples were washed twice with 2.5 mL of cell wash and resuspended in 0.5 mL of cell fix (Becton Dickinson). The whole-blood samples were resuspended in 2.5 mL of lysing solution (Becton Dickinson) to remove RBCs followed by two washes in 2.5 mL of cell wash and resuspended in 0.6 mL of cell fix. Fixed stained samples were kept overnight at 4°C in the dark before analyzing by three-color flow cytometry as previously described.[30] Samples from lysed preparations were collected on linear forward- and side-scatter axes for WBC studies, and a second set was collected on logarithmic forward- and side-scatter axes for PLT studies. RBCs were collected on linear forward- and side-scatter axes. A total of 20,000 events in appropriate forward- and side-scatter gates were collected to listmode files for each analysis. Analysis was conducted with computer software (FCS Express, DeNovoSoftware, Thornhill, Ontario, Canada) on a personal computer. Samples were gated by appropriate forward- and side-scatter patterns and additionally by CD45 expression (different WBC populations), CD41 expression (PLTs), or glycophorin A expression (RBCs). Other markers of WBC populations (e.g., CD14) were susceptible to pK digestion and were not used. Histograms were made of channel-1 (FITC) expression for negative controls (no anti-PrP MoAbs) and overlaid with histograms for channel-1 for anti-PrP (primary anti-PrP MoAbs) for each cell class: single peaks were observed in each case and their median fluorescence intensities determined by setting appropriate markers on the histograms. Net median fluorescence intensity for anti-PrP staining was obtained by subtracting the negative control (no anti-PrP MoAbs) value.[30]

Statistical analysis

Clinical groups were compared with the U test carried out with computer software (NCSS 2001 software, NCSS, Kaysville, UT). Box plots were produced by the NCSS 2001 software according to a common procedure in which boxes represent the interquartile range (IQR), the top and bottom of the box are the 25th and 75th percentiles, and the horizontal line through the box represents the median. The line and bar (whiskers) above and below the box represent the upper and lower adjacent values. The upper adjacent value is the largest observation that is not greater than the 75th percentile plus 1.5 times the IQR. The lower adjacent value is the smallest observation that is at least the 25th percentile minus 1.5 times the IQR. Outliers, shown as small circles, are those values that lie outside the upper and lower adjacent values.[31]

RESULTS

Detection of whole-blood PrP^c by DELFIA

Whole-blood samples from 10 patients with vCJD, 10 patients with sCJD, 8 neurological controls, and 29 healthy adults (1 excluded owing to lack of parallelism between dilutions) were analyzed in duplicate at five dilutions by DELFIA. For each group the median and IQR were calculated (Fig. 1A; Table 1). There is a significant decrease in the concentration of PrP^c in vCJD ($p = 0.012$) and neurological control patients ($p = 0.0004$) compared with healthy adults, but not between vCJD and sCJD, nor between neurological controls and sCJD patients. Despite the significance, there was considerable overlap between the vCJD and healthy adult control groups, which indicates that this observation would have little use as a discriminatory test for diagnosis or screening. These differences between groups were retained and continued to be significant when PrP^c concentration is not normalized for total protein but is expressed directly as units per milliliter; there remains a significant decrease in the concentration of PrP^c in vCJD ($p = 0.005$) and neurological control patients ($p = 0.0001$) when compared with healthy adults.

Detection of PLT-poor plasma PrP^c by DELFIA

PLT-poor plasma samples from 10 patients with vCJD, 10 patients with sCJD, 29 healthy adults (1 excluded owing to lack of parallelism between dilutions), and 6 samples (2 samples unavailable) from neurological controls were analyzed by DELFIA for PrP^c (Fig. 1B; Table 1). We found significant elevation in the plasma PrP^c concentration in sCJD patients when compared with both healthy adult ($p = 0.022$) and neurological control groups ($p = 0.050$), but not when compared to levels found in vCJD patients. No significant differences were found in comparisons

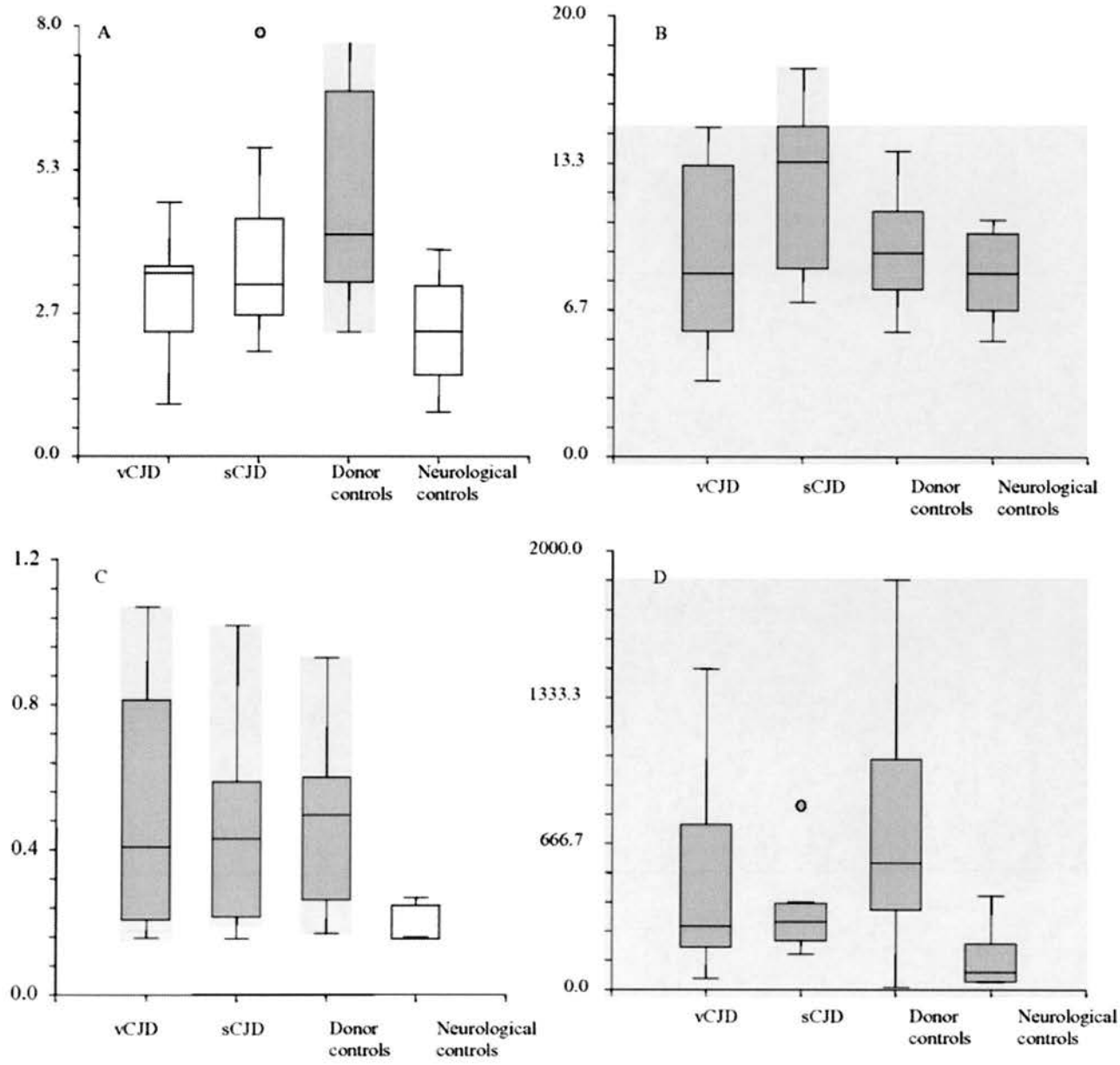

Fig. 1. The concentration of PrP^c assayed by DELFIA in separated blood components of clinical and control samples. The concentration of whole blood (A), PLT-poor plasma (B), 50 percent RBCs (C), and PLT (D) PrP^c (units per mg) assayed by DELFIA in vCJD, sCJD, and donor and neurological control groups.

between controls and vCJD groups. Again, there is considerable overlap of ranges for all groups. Significant differences between groups were not evident when data was expressed in units per milliliter of PrP^c.

Detection of RBC PrP^c by DELFIA

RBC samples from 10 vCJD patients, 9 sCJD patients, 26 healthy adults, and 7 neurological controls were analyzed by DELFIA to determine PrP^c concentration (Fig. 1C; Table 1). vCJD and sCJD patients and healthy adults all exhibited similar median values for RBC PrP^c concentration and therefore did not exhibit significant differences when compared with each other. The neurological control group showed a significant reduction in RBC PrP^c concentration when compared with vCJD patients ($p = 0.029$), sCJD patients ($p = 0.024$), and healthy adults ($p = 0.001$).

TABLE 1. Medians and range (95% confidence limits [CL]; U/mg) of separated components from different clinical and control groups as measured by DELFIA

Sample	Blood component	Number	Median (U/mg)	95% CL (U/mg)
vCJD	Whole blood	10	3.438	1.912-3.593
sCJD	Whole blood	10	3.227	2.073-5.754
Donor controls	Whole blood	29	4.16	3.376-6.635
Neurological controls	Whole blood	8	2.35	0.823-3.375
vCJD	Plasma	10	8.375	5.225-14.912
sCJD	Plasma	10	13.446	8.078-17.314
Donor controls	Plasma	29	9.316	8.038-10.624
Neurological controls	Plasma	6	8.405	5.31-10.8
vCJD	RBCs	10	0.411	0.19-1.045
sCJD	RBCs	9	0.434	0.162-0.648
Donor controls	RBCs	26	0.499	0.29-0.579
Neurological controls	RBCs	7	0.16	0.16-0.27
vCJD	PLTs	9	293.93	174.42-1150.442
sCJD	PLTs	9	316.56	213.69-400.75
Donor controls	PLTs	24	581.11	389.29-855.15
Neurological controls	PLTs	7	83.37	35.48-429.6

Detection of PLT PrP^c by DELFIA

PLT samples from 24 healthy adults, 9 patients with vCJD, 9 patients with sCJD, and 7 neurological controls were analyzed for PrP^c concentration (Fig. 1D; Table 1). Samples excluded from each group constitute those where PrP^c concentration could not be measured reproducibly across a range of dilutions. The concentration of PLT PrP^c in the sCJD samples was significantly reduced compared with levels in healthy adults ($p = 0.021$) but not against vCJD. The level of PLT PrP^c in neurological controls was the lowest of all groups, and this reduction was significant compared to levels in healthy adults ($p = 0.001$), vCJD patients ($p = 0.039$), and sCJD patient samples ($p = 0.017$).

Detection of buffy-coat PrP^c by DELFIA

Buffy-coat PrP^c expression levels were not detected at significantly high concentrations, particularly in clinical groups, to permit accurate measurement. Hence, they were excluded from the analysis.

Flow cytometry

The cell-associated PrP (net median fluorescence intensity above background) ranges for lymphocytes, PLTs, and RBCs are shown as interquartile box plots (Fig. 2) for healthy adult controls, neurological controls, sCJD cases, and vCJD cases. The only significant difference found between groups was for RBC PrP in healthy adults compared to non-CJD neurological controls ($p = 0.008$). Although PLT PrP was increased in both sCJD and vCJD cases compared to healthy adults or neurological controls, this did not reach significance. Clinical vCJD and sCJD cases were found to be as sensitive as healthy adults and non-CJD neurological patients to removal of cell-associated PrP by pK (Fig. 3). The distribution of FITC fluorescence after pK treatment was homogeneous, showing a single low peak in the FITC channel without any discernible higher peak, which might indicate the retention of protease K–resistant PrP, putative PrP^{Sc}, on any subpopulation of cells.

Results are not reported for neutrophils or monocytes because in these studies we considered the results unreliable. There appeared to be considerable nonspecific binding of fluorescence by both WBC classes especially evident in both healthy adult controls and all clinical cases, which may have been a consequence of sample age.

Stability of whole-blood PrP^c

Some samples from clinical groups took longer than 24 hours to arrive in the laboratory. To consider any effects of prolonged transit time at ambient temperature, whole-blood samples from three healthy adult controls were left at 22°C for 72 hours, and samples were removed from each control at 24-hour intervals. Samples were analyzed at each time by flow cytometry, and 1-mL samples for DELFIA analysis were stored at –80°C until analysis for the detection of whole-blood PrP^c. The expression of PrP on PLTs measured by flow cytometry at 24-hour intervals showed sequentially decreasing levels of PrP with time for each individual (not shown). This contrasted with identification of increased expression of PrP on PLTs found in a preliminary analysis of data for sCJD patients compared with healthy adult controls, and implied sample storage was not responsible for this increase in the patient group. Levels of PrP^c detected by DELFIA remained stable across 72 hour (not shown). Prolonged transit time and ambient storage temperatures had negligible effects on PrP^c concentration and are therefore unlikely to contribute to differences between sample groups.

Relationship between age and PrP^c levels

Differences between control and clinical sample groups may be affected by the age of individuals, considering that vCJD usually affects young people and sCJD older people. The concentration of PrP^c (U/mg) in whole-blood samples detected by DELFIA in CJD and control groups was plotted against age and showed that PrP^c expression is unrelated to this variable (data not shown).

Hematology of clinical and control samples

To ensure that hematologic abnormalities in blood samples from clinical patients and controls did not contribute

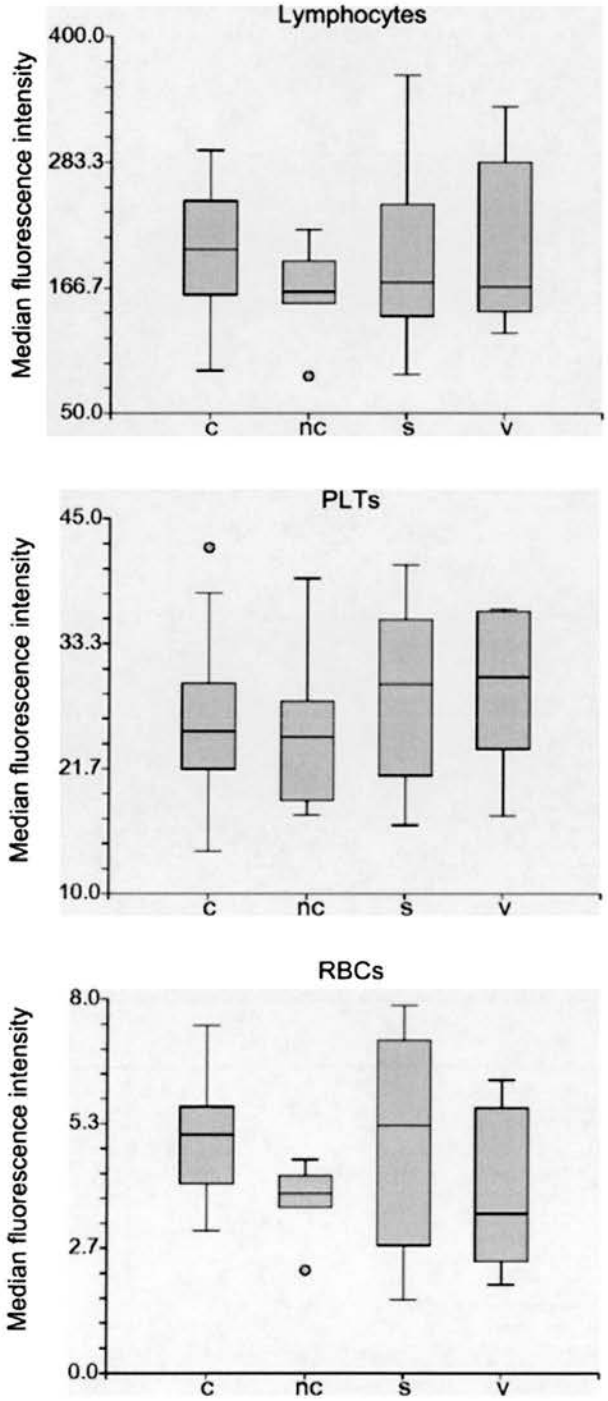

Fig. 2. Box plots showing the ranges of expression of cellular PrP shown by flow cytometry on different clinical and control groups. Expressed as median fluorescence intensity net of background, on lymphocytes, RBCs, and PLTs.

to differences in PrP concentration and expression between groups, full blood count data for clinical and neurological patients were reviewed. No gross abnormalities were detected (data not shown).

DISCUSSION

The DELFIA technique employed here, and described previously by us,[23] is well suited to the screening of whole blood and separated components of clinical and control samples for PrP^c. The assay is quick and has a high sensitivity for the detection of PrP^c. DELFIA technology is compatible with high throughput and has a high signal to background ratio, owing to background deteriorating rapidly whereas the signal has a long lifetime. Flow cytometry analysis has been used previously by us to illustrate the expression of PrP on healthy adult human blood cells.[29,30]

No previous studies have evaluated the concentration and expression of PrP in the whole blood and separated components of patients with vCJD. DELFIA analysis of separated components found that the highest levels of PrP^c associated with the PLT and plasma components and much lower levels associated with RBCs, which is in agreement with our previously described findings.[23] Levels of PrP^c in the WBC buffy-coat fractions were very low, often at the detection limit of the assay, and these levels could not be accurately calculated particularly in clinical groups, hence their exclusion from further analysis. The separation method used here was a compromise for greatest utility of archived samples and was not ideal for isolation of the PLT and buffy-coat components; cells often clumped together and proved difficult to resuspend, which may explain the large median ranges in the PLT data and the low levels of detectable PrP^c in buffy coats. These large ranges in PLT PrP^c concentrations are not supported by any large fluctuations in PLT counts as seen in hematologic data for patients and controls used in this study; the data does not show any abnormalities.

In our analysis of whole blood, we found a significant decrease in the concentration of PrP^c in vCJD and neurological control patient samples compared to healthy adults but not when compared with the sCJD group. We found significant elevation in the plasma PrP^c concentration in sCJD patients when compared with both healthy adult and neurological control groups; however, no significant differences were evident between levels in sCJD and vCJD patients. The elevation of plasma PrP^c in sCJD patients agrees with a previously published report; however, in our study a distinct finding is that levels of plasma PrP^c in sCJD patients are significantly elevated not only against healthy adults but also against the neurological control group, which was not the case in the previous published findings.[25] These differences may be a consequence of our use of neurological controls with conditions, apart from Alzheimer's disease, that were distinct from those

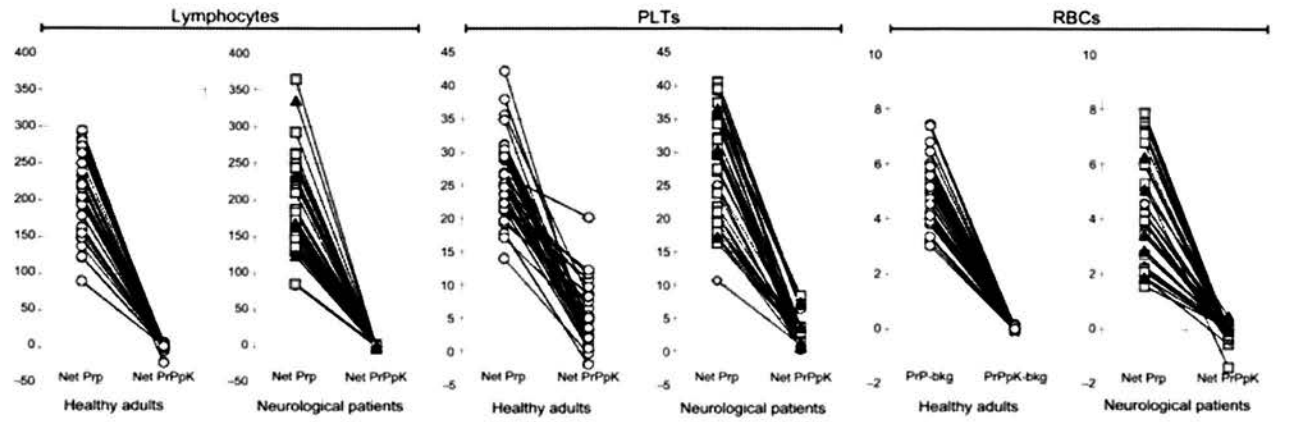

Fig. 3. Demonstration by flow cytometry of the removal of cell-surface PrP after pK treatment. The median fluorescence intensity (net of background) is plotted for cells before and after pK treatment and connected by a line for each case. (○) Healthy adult blood donors; (○) non-CJD neurological patients; (□) sCJD patients; (▲) vCJD patient; (◇) familial TSE (a single case of familial TSE was included in the study but not included in statistical analysis).

included in the previous study. We found a significant reduction in the concentration of RBC PrP^c in neurological controls compared with all other groups. We considered that the differences between groups might be a consequence of the age of patients affected by vCJD, sCJD, and neurological disorders; however, no relationship between age and PrP^c concentration was found in either control or clinical sample groups. It is possible that the reduced concentration of whole-blood PrP^c in vCJD patient samples might be due to the conversion of PrP^c to PrP^{Sc} as part of the replication process involved in disease pathogenesis. The absence of a similar reduction of whole-blood PrP^c in sCJD patients would be expected because PrP^{Sc} is largely confined to the central nervous system and replication of PrP^{Sc} in the periphery before neuroinvasion is not a feature of sCJD. Alternatively, the vCJD whole-blood reduction could be a summation of reductions in PrP^c in the plasma and PLT components, whereas in sCJD the low PLT PrP^c value is offset by the high plasma value so balancing out in whole-blood levels. It is likely that the reduced levels of whole-blood PrP^c in neurological controls is a consequence of the reduced PrP^c levels found in RBCs and PLTs in this group. To some extent the low levels of PLT PrP^c may reflect neurological disease because PrP^c levels were low in PLT samples from CJD and lowest in neurological controls but not in healthy adult controls. We are unable though to speculate on the reasons for this.

We also considered the possibility that increases in sCJD plasma PrP^c levels may be a consequence of its release from PLTs, because PLTs are known to express PrP^c on the cell surface and to store PrP^c in alpha granules, which are released into plasma in a soluble form on PLT activation.[24,32] To support this theory, we have found a significant reduction in the PLT PrP^c concentration in sCJD patient samples compared to healthy adults, implying that PLTs may have shed PrP^c into plasma. The expression of PrP on PLTs of vCJD and sCJD as determined by flow cytometry was elevated compared with healthy adult and neurological controls, and although this elevation did not reach significance, perhaps this represents up regulation of PrP before its release into plasma. Investigations into cell-surface PrP on PLTs did not implicate blood sample storage and transit as a possible cause for increased cell-surface PrP; they found cell-surface PrP on PLTs decreased with sample age.

Flow cytometry analysis demonstrated significantly less PrP expression on RBCs of neurological controls than healthy controls. This finding supports that of a reduced concentration of RBC PrP^c in this group found by DELFIA. These results would appear to indicate that there is less PrP^c per RBC compared with other groups. We are unable to explain the reason behind the finding of this reduced expression of PrP on RBCs. We did not find any evidence of a reduction in the expression of PrP on lymphocytes of patients with sCJD when compared to neurological controls in contrast to a previous report.[33] We used both 4F2 and 3F4 anti-PrP MoAbs together for these flow cytometry studies, however, whereas the previously reported study used 3F4 alone, which does not to bind to human RBCs.[29] It could be possible that MoAb 3F4 might show reduced binding to PrP^c on lymphocytes in some clinical conditions if PrP^c expression were altered or PrP^c interacted with other membrane components because of lymphocyte activation.[34,35] PrP on RBCs, PLTs, and lymphocytes was removed to background levels after pK treatment in all clinical and control groups, indicating the absence of any detectable cellular expression of abnormal disease associated forms of PrP. Our findings indicate that a reduction

in concentration of whole-blood PrP^c may be common in vCJD and other neurological diseases but not sCJD. An elevated level of plasma PrP^c may be common in sCJD. These differences between groups appear genuine, presumably reflecting differences in the disease process in the patients in these particular groups and not an artifact of age, specimen collection, storage, or analysis. Despite the significance of differences between groups, the variations in values are large and there is considerable overlap between CJD groups and control groups, which rules out the exploitation of these differences in whole blood and plasma in screening strategies. These studies expose the limitations in the use of blood PrP^c levels as a diagnostic tool. They illustrate, however, important observations on the distribution of PrP^c in the peripheral blood of CJD patients and the potential of DELFIA-based PrP assays in clinical practice. The analytical sensitivity of DELFIA-based assays used here represents a significant step toward the development of DELFIA for the detection of PrP^{Sc}, which is a much more reliable indicator of infection and is directly associated with infectivity. The current study emphasizes the need to develop assays for its detection in blood.[36,37]

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